COPY

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 August 2002 (01.08.2002)

PCT

(10) International Publication Number
WO 02/059294 A1

(51) International Patent Classification⁷: C12N 15/09, 15/63

(21) International Application Number: PCT/AU02/00073

(22) International Filing Date: 24 January 2002 (24.01.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/264,067 26 January 2001 (26.01.2001) US
60/333,743 29 November 2001 (29.11.2001) US

(71) Applicant (*for all designated States except US*): COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH O RGANISATION [AU/AU]; Limestone Avenue, Campbell, Australian Capital Territory 2601 (AU).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): WESLEY, Susan [IN/AU]; 18 Pambula Street, Kaleen, Australian Capital Territory 2617 (AU). WATERHOUSE, Peter [AU/AU]; 5 Banjine Street, O'Connor, Australian Capital Territory 2602 (AU). HELLIWELL, Christopher [AU/AU]; 25A Bingham Circuit, Kaleen, Australian Capital Territory 2617 (AU).

(74) Agents: OLIVE, Mark, R. et al.; FB RICE & CO, 139 Rathdowne Street, Carlton, Victoria 3053 (AU).

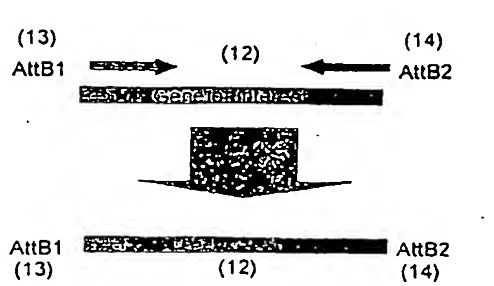
(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS AND MEANS FOR PRODUCING EFFICIENT SILENCING CONSTRUCT USING RECOMBINATIONAL CLONING



(57) Abstract: Methods and means are provided for producing chimeric nucleic acid constructs capable of producing dsRNA for silencing target nucleic acid sequences of interest using recombinational cloning.

WO 02/059294 A1

Methods and means for producing efficient silencing construct using recombinational cloning.

Field of the invention.

This invention relates to efficient methods and means for producing chimeric nucleic acid constructs capable of producing dsRNA useful for silencing target nucleic acid sequences of interest. The efficiency of the disclosed methods and means further allows high throughput analysis methods to determine the function of isolated nucleic acids, such as ESTs, without a known function and may further be put to use to isolate particular genes or nucleotide sequences from a preselected group of genes.

General

This specification contains nucleotide and amino acid sequence information prepared using PatentIn Version 3.1, presented herein after the claims. Each nucleotide sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, <210>3, etc). The length and type of sequence (DNA, protein (PRT), etc), and source organism for each nucleotide sequence, are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide sequences referred to in the specification are defined by the term "SEQ ID NO:", followed by the sequence identifier (eg. SEQ ID NO: 1 refers to the sequence in the sequence listing designated as <400>1).

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide other than Thymine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

As used herein the term "derived from" shall be taken to indicate that a specified integer may be obtained from a particular source albeit not necessarily directly from that source.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but not the exclusion of any other step or element or integer or group of elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that such prior art forms part of the common general knowledge in Australia.

<u>Background art</u>

Increasingly, the nucleotide sequence of whole genomes of organisms, including *Arabidopsis thaliana*, has been determined and as these data become available they provide a wealth of unmined information. The ultimate goal of these genome projects is to identify the biological function of every gene in the genome.

Attribution of a function to a nucleic acid with a particular nucleotide sequence can be achieved in a variety of ways. Some of the genes have been characterized directly using the appropriate assays. Others have been attributed with a tentative function through homology with (parts of) genes having a known function in other organisms. Loss-of-function mutants, obtained e.g. by tagged insertional mutagenesis have also been very informative about the role of some of these unknown genes (AzpiroLeehan and Feldmann 1997; Martienssen 1998) particularly in the large scale analysis of the yeast genome (Ross-MacDonald et al., 1999).

Structural mutants resulting in a loss-of-function may also be mimicked by interfering with the expression of a nucleic acid of interest at the transcriptional or post-transcriptional level. Silencing of genes, particularly plant genes using anti-sense or co-suppresion constructs to identify gene function, especially for a larger number of targets, is however hampered by the relatively low proportion of silenced individuals obtained, particularly those wherein the silencing level is almost complete.

Recent work has demonstrated that the silencing efficiency could be greatly improved both on quantitative and qualitative level using chimeric constructs encoding RNA capable of forming a double stranded RNA by basepairing between the antisense and sense RNA nucleotide sequences respectively complementary and homologous to the target sequences.

Fire *et al.*, 1998 describe specific genetic interference by experimental introduction of double-stranded RNA in *Caenorhabditis elegans*. The importance of these findings for functional genomics has been discussed (Wagner and Sun, 1998).

WO 99/32619 provides a process of introducing an RNA into a living cell to inhibit gene expression of a target gene in that cell. The process may be practiced ex vivo or in vivo. The RNA has a region with double-stranded structure. Inhibition is sequence-specific in that the nucleotide sequences of the duplex region of the RNA and or a portion of the target gene are identical.

Waterhouse *et al.* 1998 describe that virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and anti-sense RNA. The sense and antisense RNA may be located in one transcript that has self-complementarity.

Hamilton *et al.* 1998 describes that a transgene with repeated DNA, i.e. inverted copies of its 5' untranslated region, causes high frequency, post-transcriptional suppression of ACC-oxidase expression in tomato.

WO 98/53083 describes constructs and methods for enhancing the inhibition of a target gene within an organism which involve inserting into the gene silencing vector an inverted repeat sequence of all or part of a polynucleotide region within the vector.

WO 99/53050 provides methods and means for reducing the phenotypic expression of a nucleic acid of interest in eukaryotic cells, particularly in plant cells, by introducing chimeric genes encoding sense and antisense RNA molecules directed towards the target nucleic acid, which are capable of forming a double stranded RNA region by base-pairing between the regions with the sense and antisense nucleotide sequence or by introducing the RNA molecules themselves. Preferably, the RNA molecules comprise simultaneously both sense and antisense nucleotide sequences.

WO 99/49029 relates generally to a method of modifying gene expression and to synthetic genes for modifying endogenous gene expression in a cell, tissue or organ of a transgenic organism, in particular to a transgenic animal of plant. Synthetic genes and genetic constructs, capable of forming a dsRNA which are capable of repressing, delaying or otherwise reducing the expression of an endogenous gene or a target gene in an organism when introduced thereto are also provided.

WO 99/61631 relates to methods to alter the expression of a target gene in a plant using sense and antisense RNA fragments of the gene. The sense and antisense RNA fragments are capable of pairing and forming a double-stranded RNA molecule, thereby altering the expression of the gene. The present invention also relates to plants, their progeny and seeds thereof obtained using these methods.

WO 00/01846 provides a method of identifying DNA responsible for conferring a particular phenotype in a cell which method comprises a) constructing a cDNA or genomic library of the DNA of the cell in a suitable vector in an orientation relative to (a) promoter(s) capable of initiating transcription of the cDNA or DNA to double stranded (ds) RNA upon binding of an appropriate transcription factor to the promoter(s); b) introducing the library into one or more of cells comprising the transcription factor, and c) identifying and isolating a particular phenotype of a cell comprising the library and identifying the DNA or cDNA fragment from the library responsible for conferring the phenotype. Using this technique, it is also possible to assign function to a known DNA sequence by a) identifying homologues of the DNA sequence in a cell, b) isolating the relevant DNA homologus(s) or a fragment thereof from the cell, c) cloning the homologue or fragment thereof into an appropriate vector in an orientation relative to a suitable promoter capable of initiating transcription of dsRNA from said DNA homologue or fragment upon binding of an appropriate transcription factor to the promoter and d) introducing the vector into the cell from step a) comprising the transcription factor.

WO 00/44914 also describes composition and methods for in vivo and in vitro attenuation of gene expression using double stranded RNA, particularly in zebrafish.

WO 00/49035 discloses a method for silencing the expression of an endogenous gene in a cell, the method involving overexpressing in the cell a nucleic acid molecule of the endogenous gene and an antisense molecule including a nucleic acid molecule complementary to the nucleic acid molecule of the endogenous gene, wherein the overexpression of the nucleic acid molecule of the endogenous gene and the antisense molecule in the cell silences the expression of the endogenous gene.

Smith et al., 2000 as well as WO 99/53050 described that intron containing dsRNA further increased the efficiency of silencing.

However, the prior art has not solved the problems associated with the efficient conversion of any nucleotide sequence of interest into a chimeric construct capable of

producing a dsRNA in eukaryotic cells, particularly in plant cells, and preferably in a way amenable to the processing of large number of nucleotide sequences.

These and other problems have been solved as described hereinafter in the different embodiments and claims.

Summary of the invention.

It is an object of the invention to provide vectors comprising the following operably linked DNA fragments a) an origin of replication allowing replication in microorganisms (1), preferably bacteria; particularly Escherichia coli; b) a selectable marker region (2) capable of being expressed in microorganisms, preferably bacteria; and c) a chimeric DNA construct comprising in sequence (i) a promoter or promoter region (3) capable of being recognized by RNA polymerases of a eukaryotic cell, preferably a plant-expressible promoter; (ii) a first recombination site (4), a second recombination site (5), a third recombination site (6) and a fourth recombination site (7); and (iii) a 3' transcription terminating and polyadenylation region (8) functional in the eukaryotic cell; wherein the first recombination site (4) and the fourth recombination site (7) are capable of reacting with a same recombination site, preferably are identical, and the second recombination site (5) and the third recombination site (6), are capable of reacting with a same recombination site, preferably are identical; and wherein the first recombination site (4) and the second recombination site (5) do not recombine with each other or with a same recombination site or the third recombination site (6) and the fourth recombination site (7) do not recombine with each other or with a same recombination site. Optionally the vector may further include additional elements such as: a second selectable marker gene (9) between the first (4) and second recombination site (5) and/or a third selectable marker gene (10) between the third (6) and fourth recombination site (7) and/or a region flanked by intron processing signals (11), preferably an intron, functional in the eukaryotic cell, located between the second recombination site (5) and the third recombination site (6) and/or a fourth selectable marker gene (19), located between the second (5) and third recombination site (6) and/or left and right border T-DNA sequences flanking the chimeric DNA construct

plant, cells, preferably located between the left and the right T-DNA border sequences and/or an origin of replication capable of functioning in *Agrobacterium* spp. Selectable marker genes may be selected from the group consisting of an antibiotic resistance gene, a tRNA gene, an auxotrophic marker, a toxic gene, a phenotypic marker, an antisense oligonucleotide; a restriction endonuclease; a restriction endonuclease cleavage site, an enzyme cleavage site, a protein binding site, an a sequence complementary PCR primer. Preferably the first (4) and fourth recombination site (7) are *att*R1 comprising the nucleotide sequence of SEQ ID No 4 and the second (5) and third (6) recombination site are *att*R2 comprising the nucleotide sequence of SEQ ID No 5 or the first (4) and fourth recombination site (7) are *att*P1 comprising the nucleotide sequence of SEQ ID No 10 and the second (5) and third (6) recombination site are *att*P2 comprising the nucleotide sequence of SEQ ID No 11.

It is another objective of the invention to provide a kit comprising an acceptor vector according to invention, preferably further comprising at least one recombination protein capable of recombining a DNA segment comprising at least one of the recombination sites.

It is yet another objective of the invention to provide a method for making a chimeric DNA construct capable of expressing a dsRNA in a eukaryotic cell comprising the steps of

a) combining in vitro:
    i) an acceptor vector as herein before described;
    ii) an insert DNA, preferably a lineair or circular insert DNA, comprising a DNA segment of interest (12) flanked by
        (a) a fifth recombination site (13) which is capable of recombining with the first (4) or fourth recombination site (7) on the vector; and
        (b) a sixth recombination site (14) which is capable of recombining with the second (5) or third recombination site (6) on the vector;
    iii) at least one site specific recombination protein capable of recombining the first (4) or fourth (7) and the fifth recombination site (13) and the second (5) or third (6) and the sixth recombination site (14);

b) allowing recombination to occur in the presence of at least one recombination protein, preferably selected from Int and IHF and (ii) Int, Xis, and IHF, so as to produce a reaction mixture comprising product DNA molecules, the product DNA molecule comprising in sequence:

i) the promoter or promoter region (3) capable of being recognized by RNA polymerases of the eukaryotic cell;

ii) a recombination site (15) which is the recombination product of the first (4) and the fifth recombination site (13);

iii) the DNA fragment of interest (12);

iv) a recombination site (16) which is the recombination product of the second (4) and the sixth recombination site (14);

v) a recombination site (17) which is the recombination product of the third (5) and the sixth recombination site (14);

vi) the DNA fragment of interest in opposite orientation (12);

vii) a recombination site (18) which is the recombination product of the fourth (7) and the fifth recombination site (13); and

viii) the 3' transcription terminating and polyadenylation region (8) functional in the eukaryotic cell;

c) selecting the product DNA molecules, preferably in vivo.

The method allows that multiple insert DNAs comprising different DNA fragments of interest are processed simultaneously.

The invention also provides a method for preparing a eukaryotic non-human organism, preferably a plant, wherein the expression of a target nucleic acid of interest is reduced or inhibited, the method comprising:

a) preparing a chimeric DNA construct capable of expressing a dsRNA in cells of the eukaryotic non-human organism according to methods of the invention;

b) introducing the chimeric DNA construct in cells of the eukaryotic non-human organism; and

c) isolating the transgenic eukaryotic organism

It is also an objective of the invention to provide a method for isolating a nucleic acid molecule involved in determining a particular trait

a) preparing a library of chimeric DNA constructs capable of expressing a dsRNA in cells of the eukaryotic non-human organism according to any one of the methods of the invention;

b) introducing individual representatives of the library of chimeric DNA constructs in cells of the eukaryotic non-human organism;

c) isolating a eukaryotic organism exhibiting the particular trait; and isolating the nucleic acid molecule.

The invention also provides a eukaryotic non-human organism, preferably a plant comprising a chimeric DNA construct obtainable through the methods of the invention.

Brief description of the figures.

Figure 1. Schematic representation of vectors and method used in a preferred embodiment of the invention.

Figure 1A: A nucleic acid of interest (12) is amplified by PCR using primers comprising two different recombination sites (13, 14) which cannot react with each other or with the same other recombination site. This results in "insert DNA" wherein the nucleic acid of interest (12) is flanked by two different recombination sites (13, 14).

Figure 1B. Using at least one recombination protein, the insert DNA is allowed to recombine with the acceptor vector between the recombination sites, whereby the first (4) and fourth recombination site (7) react with one of the recombination sites (13) flanking the PCR amplified DNA of interest (12) and the second (5) and third (6) recombination site on the acceptor vector recombine with the other recombination site (14) flanking the DNA of interest (12). The desired product DNA can be isolated by selecting for loss of the selectable marker genes (9) and (10) located between respectively the first (4) and second (5) recombination sites and the third (6) and fourth (7) recombination sites. Optionally, an additional selectable marker gene may

be included between the second (5) and third (6) recombination site to allow selection for the presence of this selectable marker gene and consequently for the optional intron sequence, which is flanked by functional intron processing signal sequences (11). The acceptor vector, as well as the product vector further comprises a origin of replication (Ori; (1)) and a selectable marker gene (2) to allow selection for the presence of the plasmid.

This result in a chimeric DNA construct with the desired configuration comprising a eukaryotic promoter region (3); a recombination site (15) produced by the recombination between recombination sites (4) and (13); a first copy of the DNA of interest (12); a recombination site (16) produced by the recombination between recombination sites (5) and (14); optionally an intron sequence flanked by intron processing signals (11); a recombination site (17) produced by the recombination between recombination sites (6) and (14); a second copy of the DNA of interest (12) in opposite orientation to the first copy of the DNA of interest; a recombination site (18) produced by the recombination between recombination sites (7) and (13); a eukaryotic transcription terminator and polyadenylation signal (8).

Figure 2A: A nucleic acid of interest (12) is amplified by PCR using primers comprising two different recombination sites which upon recombination with the recombination sites on an intermediate vector (Figure 2B) will yield recombination sites compatible with the first (4) and fourth (5) and with the second (6) and third (7) recombination site on the acceptor vector respectively.

Figure 2B: The insert DNA obtained in Figure 2A is allowed to recombine with the intermediate vector in the presence of at least one recombination protein to obtain an intermediate DNA wherein the DNA of interest (12) is flanked by two different recombination sites (13, 14) and which further comprises an origin of replication (1) and a selectable marker gene (2).

Figure 2C: The intermediate DNA is then allowed to recombine with the acceptor vector using at least one second recombination protein (basically as described for Figure 1B).

Figure 3: Schematic representation of the acceptor vector "pHELLSGATE"

Figure 4: Schematic representation of the acceptor vectors "pHELLSGATE 8" "pHELLSGATE 11" and "pHELLSGATE 12".

Detailed description of preferred embodiments.

The current invention is based on the unexpected finding by the inventors that recombinational cloning was an efficient one-step method to convert a nucleic acid fragment of interest into a chimeric DNA construct capable of producing a dsRNA transcript comprising a sense and antisense nucleotide sequence capable of being expressed in eukaryotic cells. The dsRNA molecules are efficient effectors of gene-silencing. These methods improves the efficiency problems previously encountered to produce chimeric DNAs with long inverted repeats.

Thus, in a first embodiment, the invention provides a method for making a chimeric DNA construct or chimeric gene capable of expressing an RNA transcript in a eukaryotic cell , the RNA being capable of internal basepairing between a stretch of nucleotides corresponding to a nucleic acid of interest and its complement (i.e. the stretch of nucleotides in inverted orientation) located elsewhere in the transcript (and thus forming a hairpin RNA) comprising the following steps:

1. Providing an "acceptor vector" comprising the following operably linked DNA fragments:
   a) an origin of replication allowing replication in a host cell (1),
   b) a selectable marker region (2) capable of being expressed in the host cell; and
   c) a chimeric DNA construct comprising in sequence:
      i) a promoter or promoter region (3) capable of being recognized by RNA polymerases of a eukaryotic cell;
      ii) a first recombination site (4), a second recombination site (5), a third recombination site (6) and a fourth recombination site (7) whereby
         (1) the first (4) and fourth recombination site (7) are capable of reacting with the same other recombination site and preferably are identical to each other;

(2) the second (5) and third (6) recombination site are also capable of reacting with the same other recombination site and preferably are identical to each other

(3) the first (4) and second (5) recombination site do not recombine with each other or with the same other recombination site; and

(4) the third (6) and fourth (7) recombination site do not recombine with each other or with the same other recombination site; and

iii) a 3' transcription terminating and polyadenylation region (8) functional in a eukaryotic cell.

2. Providing an "insert DNA" comprising the DNA segment of interest (12) flanked by
   a) a fifth recombination site (13) which is capable of recombining with the first(4) or fourth (7) recombination site but preferably not with the second (5) or third (6) recombination site;
   b) a sixth recombination site (14) which is capable of recombining with the second (5) or third (6) recombination site but preferably not with the first (4) or fourth (7) recombination site.

3. Combining *in vitro* the insert DNA and the acceptor vector in the presence of at least one specific recombination protein and allowing the recombination to occur to produce a reaction mixture comprising inter alia "product DNA" molecules which comprise in sequence
   i) the promoter or promoter region (3) capable of being recognized by RNA polymerases of a eukaryotic cell;
   ii) a recombination site (15) which is the recombination product of the first (4) and fifth recombination site (13);
   iii) a first copy of the DNA fragment of interest (12);
   iv) a recombination site (16) which is the recombination product of the second (4) and the sixth recombination site (14);
   v) a recombination site (17) which is the recombination product of the third (5) and the sixth recombination site (14);
   vi) a second copy of the DNA fragment of interest in opposite orientation (12) with regard to the first copy ;

vii) a recombination site (18) which is the recombination product of the fourth (7) and the fifth recombination site (13); and

viii) a 3' transcription terminating and polyadenylation region (8) functional in a eukaryotic cell;

4. Selecting the product DNA molecules.

This method is schematically outlined in Figure 1, with non-limiting examples of recombination sites and selectable markers.

As used herein, a "host cell" is any prokaryotic or eukaryotic organism that can be a recipient for the acceptor vector or the product DNA. Conveniently, the host cell will be a *Escherichia coli* strain commonly used in recombinant DNA methods.

A "recombination protein" is used herein to collectively refer to site specific recombinases and associated proteins and/or co-factors. Site specific recombinases are enzymes that are present in some viruses and bacteria and have been characterized to have both endonuclease and ligase properties. These recombinases (along with associated proteins in some cases) recognize specific sequences of bases in DNA and exchange the DNA segments flanking those segments. Various recombination proteins are described in the art(see WO 96/40724 herein incorporated by reference in its entirety, at least on page 22 to 26).

Examples of such recombinases include Cre from bacteriophage P1 and Integrase from bacteriophage lambda.

Cre is a protein from bacteriophage P1 (Abremski and Hoess, 1984) which catalyzes the exchange between 34 bp DNA sequences called *lox*P sites (see Hoess et al., 1986. Cre is available commercially (Novagen, Catalog 69247-1).

Integrase (Int) is a protein from bacteriophage lambda which mediates the integration of the lambda genome into the *E. coli* chromosome. The bacteriophage lambda Int recombinational proteins promote irreversible recombination between its substrate *att*

sites as part of the formation or induction of a lysogenic state. Reversibility of the recombination reactions results from two independent pathways for integrative or excisive recombination. Cooperative and competitive interactions involving four proteins (Int, Xis, IHF and FIS) determine the direction of recombination. Integrative recombination involves the Int and IHF proteins and *attP* (240bp) and *att*B (25b) recombination sites. Recombination results in the formation of two new sites: *att*L and *att*R. A commercial preparation comprising Int and IHF proteins is commercially available (BP clonase™ ; Life Technologies). Excisive recombination requires Int, IHF, and Xis and sites *att*L and *att*R to generate *att*P and *att*B. A commercial preparation comprising Int, IHF and Xis proteins is commercially available (LR clonase™ ; Life Technologies).

A "recombination site" as used herein refers to particular DNA sequences, which a recombinase and possibly associated proteins recognizes and binds. The recombination site recognized by Cre recombinase is *lox*P which is a 34 base pair sequence comprised of two 13 base pair inverted repeats (serving as recombinase binding sites) flanking an 8 base pair core sequence. The recombination sites *att*B, *att*P, *att*L and *att*R are recognized by lambda integrase. *Att*B is an approximately 25 base pair sequence containing two 9 base pair core-type Int binding sites and a 7 base pair overlap region. *Att*P is an approximately 240 base pair sequence containing core-type Int binding sites and arm-type Int binding sites as well as sites for auxiliary proteins IHF, FIS and Xis (Landy 1993). Each of the *att* sites contains a 15 bp core sequence with individual sequence elements of functional significance lying within, outside and across the boundaries of this common core (Landy, 1989) Efficient recombination between the various att sites requires that the sequence of the central common region is substantially identical between the recombining partners. The exact sequence however is modifiable as disclosed in WO 96/40724 and the variant recombination sites selected from

i) *att*B1: AGCCTGCTTTTTTGTACAAACTTGT (SEQ ID No 1);
ii) *att*B2: AGCCTGCTTTCTTGTACAAACTTGT (SEQ ID No 2);
iii) *att*B3: ACCCAGCTTTCTTGTACAAACTTGT (SEQ ID No 3);
iv) *att*R1: GTTCAGCTTTTTTGTACAAACTTGT (SEQ ID No 4);
v) *att*R2: GTTCAGCTTTCTTGTACAAACTTGT (SEQ ID No 5);

vi) *att*R3: GTTCAGCTTTCTTGTACAAAGTTGG (SEQ ID No 6);

vii) *att*L1: AGCCTGCTTTTTTGTACAAAGTTGG (SEQ ID No 7);

viii) *att*L2: AGCCTGCTTTCTTGTACAAAGTTGG (SEQ ID No 8);

ix) *att*L3: ACCCAGCTTTCTTGTACAAAGTTGG (SEQ ID No 9);

x) *att*P1: GTTCAGCTTTTTTGTACAAAGTTGG (SEQ ID No 10); or

xi) *att*P2,P3: GTTCAGCTTTCTTGTACAAAGTTGG (SEQ ID No 11)

allow more flexibility in the choice of suitable pairs or recombination sites which are capable to recombine (as indicated by their index number).

It will be clear to the skilled artisan that a correspondence is required between the recombination site(s) used and the recombination proteins used.

In one embodiment the following combinations of recombination sites for the acceptor vector are present in the acceptor vector:

- the first (4) and fourth (7) recombination sites are identical and comprise *att*P1 comprising the nucleotide sequence of SEQ ID No 10 and the second (5) and third (6) recombination site are also identical and comprise *att*P2 comprising the nucleotide sequence of SEQ ID No 11; or
- the first (4) and fourth (7) recombination sites are identical and comprise *att*R1 comprising the nucleotide sequence of SEQ ID No 4 and the second (5) and third (6) recombination site are also identical and comprise *att*R2 comprising the nucleotide sequence of SEQ ID No 5; and

the following combinations of recombination sites for the insert DNA are used:

- the fifth (13) recombination site comprises *att*B1 comprising the nucleotide sequence of SEQ ID No 1 and the sixth (14) recombination site comprises *att*B2 comprising the nucleotide sequence of SEQ ID No 2, the combination being suitable for recombination with the first acceptor vector mentioned above; or
- the fifth (13) recombination site comprises *att*L1 comprising the nucleotide sequence of SEQ ID No 7 and the sixth (14) recombination site comprises *att*L2 comprising the nucleotide sequence of SEQ ID No 8, the combination being suitable for recombination with the second acceptor vector mentioned above.

It has been unexpectedly found that product DNA molecules (resulting from recombination between the above mentioned second acceptor vector with attR recombination sites (such as pHELLSGATE 8) and insert DNA flanked by attL recombination sites) wherein the gene inserts in both orientations are flanked by attB recombination sites are more effective in silencing of the target gene(both quantitatively and qualitatively) than product DNA molecules (resulting from recombination between the above mentioned first acceptor vector with attP recombination sites (such as pHELLSGATE or pHELLSGATE 4) and insert DNA flanked by attB recombination sites) wherein the gene inserts in both orientations are flanked by attL recombination sites. Although not intending to limit the invention to a particular mode of action it is thought that the greater length of the attL sites and potential secondary structures therein may act to inhibit transcription yielding the required dsRNA to a certain extent. However, acceptor vectors such as the above mentioned first acceptor vectors with attP sites may be used when target gene silencing to a lesser extent would be useful or required.

The dsRNA obtained by the chimeric DNA construct made according to the invention may be used, to silence a nucleic acid of interest, i.e. reduce its phenotypic expression, in a eukaryotic organism, particularly a plant, either directly or by transcription of the chimeric DNA construct in the cells of the eukaryotic organism. When this is the case, the following considerations may apply.

The length of the nucleic acid of interest (12) may vary from about 10 nucleotides (nt) up to a length equaling the length (in nucleotides) of the target nucleic acid whose phenotypic expression is to be reduced. Preferably the total length of the sense nucleotide sequence is at least 10 nt, or at least 19 nt or at least 21 nt or at least 25 nt, or at least about 50 nt, or at least about 100 nt, or at least about 150 nt, or at least about 200 nt, or at least about 500 nt. It is expected that there is no upper limit to the total length of the sense nucleotide sequence, other than the total length of the target nucleic acid. However for practical reason (such as e.g. stability of the chimeric genes) it is expected that the length of the sense nucleotide sequence should not exceed 5000 nt, particularly should not exceed 2500 nt and could be limited to about 1000 nt.

It will be appreciated that the longer the total length of the nucleic acid of interest (12), the less stringent the requirements for sequence identity between the nucleic acid of interest and the corresponding sequence in the target gene. Preferably, the nucleic acid of interest should have a sequence identity of at least about 75% with the corresponding target sequence, particularly at least about 80 %, more particularly at least about 85%, quite particularly about 90%, especially about 95%, more especially about 100%, quite especially be identical to the corresponding part of the target nucleic acid. However, it is preferred that the nucleic acid of interest always includes a sequence of about 10 consecutive nucleotides, particularly about 25 nt, more particularly about 50 nt, especially about 100 nt, quite especially about 150 nt with 100% sequence identity to the corresponding part of the target nucleic acid. Preferably, for calculating the sequence identity and designing the corresponding sense sequence, the number of gaps should be minimized, particularly for the shorter sense sequences.

For the purpose of this invention, the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e. a position in an alignment where a residue is present in one sequence but not in the other is regarded as a position with non-identical residues. The alignment of the two sequences is performed by the Needleman and Wunsch algorithm (Needleman and Wunsch 1970) The computer-assisted sequence alignment above, can be conveniently performed using standard software program such as GAP which is part of the Wisconsin Package Version 10.1 (Genetics Computer Group, Madision, Wisconsin, USA) using the default scoring matrix with a gap creation penalty of 50 and a gap extension penalty of 3. Sequences are indicated as "essentially similar" when such sequence have a sequence identity of at least about 75%, particularly at least about 80 %, more particularly at least about 85%, quite particularly about 90%, especially about 95%, more especially about 100%, quite especially are identical. It is clear than when RNA sequences are the to be essentially similar or have a certain degree of sequence identity with DNA sequences, thymine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence.

The "insert DNA" may conveniently be provided using DNA amplification procedures, such as PCR, of the nucleic acid of interest, using as primers oligonucleotide sequences incorporating appropriate recombination sites as well as oligonucleotide sequences appropriate for the amplification of the nucleic acid of interest. However, alternative methods are available in the art to provide the nucleic acid of interest with the flanking recombination sites, including but not limited to covalently linking oligonucleotides or nucleic acid fragments comprising such recombination sites to the nucleic acid(s) of interest using ligase(s).

The providing of the appropriate flanking recombination sites to the nucleic acid may also proceed in several steps. E.g. in a first step the flanking sites provided to the nucleic acid of interest may be such that upon recombination with the recombination sites in an intermediate vector new recombination sites are created flanking the nucleic acid of interest, now compatible for recombination with the acceptor vector. This scheme is outlined in Figure 2, with non-limiting examples of recombination sites and selectable markers. It goes without saying that the insert DNA may be in a circular form or in a linear form.

As used herein, an "origin of replication" is a DNA fragment which allows replication of the acceptor vector in microorganisms, preferably bacteria, particularly *E. coli* strains, and ensures that upon multiplication of the microorganism, the daughter cells receive copies of the acceptor vector.

"Selectable marker (gene)" is used herein to indicate a DNA segment which allows to select or screen for the presence or absence of that DNA segment under suitable conditions. Selectable markers include but are not limited to

(1) DNA segments that encode products which provide resistance against otherwise toxic compounds (e.g. antibiotic resistance genes, herbicide resistance genes)

(2) DNA segments encoding products which are otherwise lacking in the recipient cell (e.g. tRNA genes, auxotrophic markers)

(3) DNA segments encoding products which suppress the activity of a gene product;

(4) DNA segments encoding products which can readily be identified (e.g. β-galactosidase, green fluorescent protein (GFP), β-glucuronidase (GUS));

(5) DNA segments that bind products which are otherwise detrimental to cell survival and/or function;

(6) DNA segments that are capable of inhibiting the activity of any of the DNA segments described in Nos 1 to 5 (e.g. antisense oligonucleotides);

(7) DNA segments that bind products that modify a substrate (e.g. restriction endonuclease);

(8) DNA segments that can be used to isolate a desired molecule (e.g. specific protein binding sites);

(9) DNA segments that encode a specific nucleotide seqeunce which can be otherwise non-functional (e.g. for PCR amplification of subpopulations of molecules;

(10) DNA segments, which when absent, directly or indirectly confer sensitivity to particular compound(s);

(11) DNA segments, which when absent, directly or indirectly confer resistance to particular compound(s);

Preferred first selectable markers (2) are antibiotic resistance genes. A large number of antibiotic resistance genes, particularly which can be used in bacteria, are available in the art and include but are not limited to aminoglycoside phosphotransferase I and II, chloramphenicol acetyltransferase, beta-lactamase, aminoglycoside adenosyltransferase.

Preferred second selectable marker (9) and third selectable markers (10) are selectable markers allowing a positive selection when absent or deleted after recombination (i.e. in the product DNA) such as but not limited to *ccd*B gene the product of which interferes with *E. coli* DNA gyrase and thereby inhibits growth of most *E. coli* strains. Preferably, the second and third marker are identical.

In one embodiment of the invention, the acceptor comprises a fourth selectable marker (19) between the second (5) and third (6) recombination site, preferably a marker allowing positive selection for the presence thereof, such as a antibiotic resistance gene, e.g. chloramphenicol resistance gene. Preferably, the fourth selectable marker should be different from first selectable marker and different from the second and third selectable marker. The presence of a fourth selectable marker allows to select or screen for the retention of the DNA region between the second (5) and third (6) recombination site in the product DNA, thereby increasing the efficiency with which the desired product DNAs having the nucleic acid of interest cloned in inverted repeat and operably linked to eukaryotic expression signals may be obtained. However, it has been found that with most of the acceptor vectors tested, the presence of a selectable marker is not required and has little influence on the ratio of expected and desired product DNA molecules (which usually exceeds about 90% of obtained product DNA molecules) to undesired product DNA molecules.

It goes without saying that a person skilled in the art has a number of techniques available for recognizing the expected and desired product DNA molecules, such as but not limited to restriction enzyme digests or even determining the nucleotide sequence of the recombination product.

In another embodiment of the invention, the acceptor vector further comprises a pair of intron processing signals (11) or an intron sequence functional in the eukaryotic cell, preferably located between the second (5) and third (6) recombination site. However, the pair of intron processing signals or the intron may also be located elsewhere in the chimeric construct between the promoter or promoter region (3) and the terminator region (8). As indicated in the background art, this will improve the efficiency with which the chimeric DNA construct encoding the dsRNA will be capable of reducing the phenotypic expression of the target gene in the eukaryotic cell. A particularly preferred intron functional in cells of plants is the *pdk* intron (*Flaveria trinervia* pyruvate orthophosphate dikinase intron 2 ; see WO99/53050 incorporated by reference). The fourth selectable marker (19) may be located between the intron processing signals or within the intron (if these are located between the

second and third recombination site), but may also be located adjacent to the intron processing signals or the intron.

A person skilled in the art will recognize that the product DNA molecules resulting from a recombination with an acceptor vector as herein described which comprise a region between the second (5) and third (6) recombination will fall into two classes which can be recognized by virtue of the orientation of that intervening region. In the embodiments wherein the acceptor vector also comprises an intron, the different orientation may necessitate an additional step of identifying the correct orientation. To avoid this additional step, the acceptor vector may comprise an intron which can be spliced out independent of its orientation (such as present in pHELLSGATE 11) or the acceptor vector may comprise an spliceable intron in both orientations (such as present in pHELLSGATE 12).

As used herein, the term "promoter" denotes any DNA which is recognized and bound (directly or indirectly) by a DNA-dependent RNA-polymerase during initiation of transcription. A promoter includes the transcription initiation site, and binding sites for transcription initiation factors and RNA polymerase, and can comprise various other sites (e.g., enhancers), at which gene expression regulatory proteins may bind.

The term "regulatory region", as used herein, means any DNA, that is involved in driving transcription and controlling (i.e., regulating) the timing and level of transcription of a given DNA sequence, such as a DNA coding for a protein or polypeptide. For example, a 5' regulatory region (or "promoter region") is a DNA sequence located upstream (i.e., 5') of a coding sequence and which comprises the promoter and the 5'-untranslated leader sequence. A 3' regulatory region is a DNA sequence located downstream (i.e., 3') of the coding sequence and which comprises suitable transcription termination (and/or regulation) signals, including one or more polyadenylation signals.

As used herein, the term "plant-expressible promoter" means a DNA sequence which is capable of controlling (initiating) transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable

of directing transcription in a plant cell, i.e., certain promoters of viral or bacterial origin such as the CaMV35S, the subterranean clover virus promoter No 4 or No 7, or T-DNA gene promoters but also tissue-specific or organ-specific promoters including but not limited to seed-specific promoters (e.g., WO89/03887), organ-primordia specific promoters (An et al., 1996), stem-specific promoters (Keller et al., 1988), leaf specific promoters (Hudspeth et al., 1989), mesophyl-specific promoters (such as the light-inducible Rubisco promoters), root-specific promoters (Keller et al.,1989), tuber-specific promoters (Keil et al., 1989), vascular tissue specific promoters (Peleman et al., 1989), stamen-selective promoters (WO 89/10396, WO 92/13956), dehiscence zone specific promoters (WO 97/13865) and the like.

The acceptor vector may further comprise a selectable marker for expression in a eukaryotic cell. Selectable marker genes for expression in eukaryotic cells are well known in the art, including but not limited to chimeric marker genes. The chimeric marker gene can comprise a marker DNA that is operably linked at its 5' end to a promoter, functioning in the host cell of interest, particularly a plant-expressible promoter, preferably a constitutive promoter, such as the CaMV 35S promoter, or a light inducible promoter such as the promoter of the gene encoding the small subunit of Rubisco; and operably linked at its 3' end to suitable plant transcription 3' end formation and polyadenylation signals. It is expected that the choice of the marker DNA is not critical, and any suitable marker DNA can be used. For example, a marker DNA can encode a protein that provides a distinguishable colour to the transformed plant cell, such as the A1 gene (Meyer *et al.*, 1987), can provide herbicide resistance to the transformed plant cell, such as the *bar* gene, encoding resistance to phosphinothricin (EP 0,242,246), or can provide antibiotic resistance to the transformed cells, such as the *aac(6')* gene, encoding resistance to gentamycin (WO94/01560).

The acceptor vector may also further comprise left and right T-DNA border sequences flanking the chimeric DNA construct, and may comprise an origin of replication functional in *Agrobacterium spp.* and/or a DNA region of homology with a helper Ti-plasmid as described in EP 0 116 718.

The efficiency and ease by which any nucleic acid of interest may be converted into a chimeric DNA construct comprising two copies of the nucleic acid of interest in inverted repeat and operably linked to eukaryotic 5' and 3' regulatory regions using the means and methods according to the invention, makes these particularly apt for automation and high throughput analysis.

It will be clear to the person skilled in the art that the acceptor vectors as hereinbefore described can be readily adapted to provide a vector which can be used to produce *in vitro* large amounts of double stranded RNA or RNAi comprising a complementary sense and antisense portion essentially similar to a target gene of choice as described elsewhere in this application, by exchanging the promoter capable of being expressed in a eukaryotic cell for a promoter recognized by any RNA polymerase. Very suitable promoters to this end are the promoters recognized by bacteriophage single subunit RNA polymerases such as the promoters recognized by bacteriophage single subunit RNA polymerase such as the RNA polymerases derived from the E. coli phages T7, T3, □I, □II, W31, H, Y, A1, 122, cro, C21, C22, and C2; Pseudomonas putida phage gh-1; Salmonella typhimurium phage SP6; Serratia marcescens phage IV; Citrobacter phage ViIII; and Klebsiella phage No.11 [Hausmann, Current Topics in Microbiology and Immunology, 75: 77-109 (1976); Korsten et al., J. Gen Virol. 43: 57-73 (1975); Dunn et al., Nature New Biology, 230: 94-96 (1971); Towle et al., J. Biol. Chem. 250: 1723-1733 (1975); Butler and Chamberlin, J. Biol. Chem., 257: 5772-5778 (1982)]. Examples of such promoters are a T3 RNA polymerase specific promoter and a T7 RNA polymerase specific promoter, respectively. A T3 promoter to be used as a first promoter in the CIG can be any promoter of the T3 genes as described by McGraw et al, Nucl. Acid Res. 13: 6753-6766 (1985). Alternatively, a T3 promoter may be a T7 promoter which is modified at nucleotide positions -10, -11 and -12 in order to be recognized by T3 RNA polymerase [(Klement et al., J. Mol. Biol. 215, 21-29(1990)]. A preferred T3 promoter is the promoter having the "consensus" sequence for a T3 promoter, as described in US Patent 5,037,745. A T7 promoter which may be used according to the invention, in combination with T7 RNA polymerase, comprises a promoter of one of the T7 genes as described by Dunn and Studier, J. Mol. Biol. 166: 477-535 (1983). A preferred T7 promoter is the promoter having the "consensus"

sequence for a T7 promoter, as described by Dunn and Studier (supra). Thus, the invention also provides an acceptor vector comprising

a) origin of replication allowing replication in a host cell (1),

b) a selectable marker region (2) capable of being expressed in the host cell; and

c) a chimeric DNA construct comprising in sequence:

  i) a promoter or promoter region (3) capable of being recognized by a bacteriophage single subunit RNA polymerase;

  ii) a first recombination site (4), a second recombination site (5), a third recombination site (6) and a fourth recombination site (7) whereby

    (1) the first (4) and fourth recombination site (7) are capable of reacting with the same other recombination site and preferably are identical to each other;

    (2) the second (5) and third (6) recombination site are also capable of reacting with the same other recombination site and preferably are identical to each other

    (3) the first (4) and second (5) recombination site do not recombine with each other or with the same other recombination site; and

    (4) the third (6) and fourth (7) recombination site do not recombine with each other or with the same other recombination site; and

    (5) a 3' transcription terminating and polyadenylation region (8) functional in a eukaryotic cell.

The acceptor vector may be used to convert a DNA fragment of interest into an inverted repeat structure as described elsewhere in the application and dsRNA can be produced in large amounts by contacting the acceptor vector DNA with the appropriate bacteriophage single subunit RNA polymerase under conditions well known to the skilled artisan. The so-produced dsRNA can then be used for delivery into cells prone to gene silencing, such as plant cells, fungal cells or animal cells. dsRNA may be introduced in animal cells via liposomes or other transfection agents (e.g. Clonfection transfection reagent or the CalPhos Mammalian transfection kit from ClonTech) and could be used for methods of treatment of animals, including humans, by silencing the appropriate target genes.

The acceptor vectors may also be equipped with any prokaryotic promoter suitable for expression of dsRNA in a particular prokaryotic host. The prokaryotic host can be used as a source of dsRNA, e.g. by feeding it to an animal, such as a nematode, in which the silencing of the target gene is envisioned.

The promoter capable of expression in eukaryotic cell may also be a promoter capable of expression in a mammalian cell and vectors according to the invention may transiently be delivered using a retroviral delivery system or other animal transfection system.

In another embodiment of the invention, a method is provided for making a eukaryotic organism, particularly a plant, wherein the phenotypic expression of a target nucleic acid of interest is reduced or inhibited, comprising the steps of preparing a chimeric DNA construct comprising a nucleic acid of interest (12) comprising a nucleotide sequence of at least 19 bp or 25 bp having at least 70% sequence identity to the target nucleic acid of interest and capable of expressing a dsRNA in cells of the eukaryotic organism, particularly a plant according to the methods of the current invention and introducing the chimeric DNA construct in cells of the eukaryotic organism, and isolating eukaryotic organism transgenic for the chimeric DNA construct.

As used herein, "phenotypic expression of a target nucleic acid of interest" refers to any quantitative trait associated with the molecular expression of a nucleic acid in a host cell and may thus include the quantity of RNA molecules transcribed or replicated, the quantity of post-transcriptionally modified RNA molecules, the quantity of translated peptides or proteins, the activity of such peptides or proteins.

A "phenotypic trait" associated with the phenotypic expression of a nucleic acid of interest refers to any quantitative or qualitative trait, including the trait mentioned, as well as the direct or indirect effect mediated upon the cell, or the organism containing that cell, by the presence of the RNA molecules, peptide or protein, or posttranslationally modified peptide or protein. The mere presence of a nucleic acid in a host cell, is not considered a phenotypic expression or a phenotypic trait of that

nucleic acid, even though it can be quantitatively or qualitatively traced. Examples of direct or indirect effects mediated on cells or organisms are, e.g., agronomically or industrial useful traits, such as resistance to a pest or disease; higher or modified oil content etc.

As used herein, "reduction of phenotypic expression" refers to the comparison of the phenotypic expression of the target nucleic acid of interest to the eucaryotic cell in the presence of the RNA or chimeric genes of the invention, to the phenotypic expression of the target nucleic acid of interest in the absence of the RNA or chimeric genes of the invention. The phenotypic expression in the presence of the chimeric RNA of the invention should thus be lower than the phenotypic expression in absence thereof, preferably be only about 25%, particularly only about 10%, more particularly only about 5% of the phenotypic expression in absence of the chimeric RNA, especially the phenotypic expression should be completely inhibited for all practical purposes by the presence of the chimeric RNA or the chimeric gene encoding such an RNA.

A reduction of phenotypic expression of a nucleic acid where the phenotype is a qualitative trait means that in the presence of the chimeric RNA or gene of the invention, the phenotypic trait switches to a different discrete state when compared to a situation in which such RNA or gene is absent. A reduction of phenotypic expression of a nucleic acid may thus, *i.a.* be measured as a reduction in transcription of (part of) that nucleic acid, a reduction in translation of (part of) that nucleic acid or a reduction in the effect the presence of the transcribed RNA(s) or translated polypeptide(s) have on the eucaryotic cell or the organism, and will ultimately lead to altered phenotypic traits. It is clear that the reduction in phenotypic expression of a target nucleic acid of interest, may be accompanied by or correlated to an increase in a phenotypic trait.

As used herein a "target nucleic acid of interest" refers to any particular RNA molecule or DNA sequence which may be present in a eucaryotic cell, particularly a plant cell whether it is an endogenous nucleic acid, a transgenic nucleic acid, a viral nucleic acid, or the like.

Methods for making transgenic eukaryotic organisms, particularly plants are well known in the art. Gene transfer can be carried out with a vector that is a disarmed Ti-plasmid, comprising a chimeric gene of the invention, and carried by *Agrobacterium*. This transformation can be carried out using the procedures described, for example, in EP 0 116 718. A particular kind of *Agrobacterium* mediated transformation methods are the so-called *in planta* methods, which are particularly suited for *Arabidopsis* spp. transformation (e.g. Clough and Bent 1998). Alternatively, any type of vector can be used to transform the plant cell, applying methods such as direct gene transfer (as described, for example, in EP 0 233 247), pollen-mediated transformation (as described, for example, in EP 0 270 356, WO85/01856 and US 4,684,611), plant RNA virus-mediated transformation (as described, for example, in EP 0 067 553 and US 4,407,956), liposome-mediated transformation (as described, for example, in US 4,536,475), and the like. Other methods, such as microprojectile bombardment, as described for corn by Fromm *et al.* (1990) and Gordon-Kamm *et al.* (1990), are suitable as well. Cells of monocotyledonous plants, such as the major cereals, can also be transformed using wounded and/or enzyme-degraded compact embryogenic tissue capable of forming compact embryogenic callus, or wounded and/or degraded immature embryos as described in WO92/09696. The resulting transformed plant cell can then be used to regenerate a transformed plant in a conventional manner.

The obtained transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the chimeric gene for reduction of the phenotypic expression of a nucleic acid of interest of the invention in other varieties of the same or related plant species, or in hybrid plants. Seeds obtained from the transformed plants contain the chimeric genes of the invention as a stable genomic insert.

In another embodiment the invention provides a method for isolating a nucleic acid molecule involved in determining a particular phenotypic trait of interest. The method involves the following steps:

a) preparing a library of chimeric DNA constructs capable of expressing a dsRNA in cells of the eukaryotic non-human organism using the methods and means described in the current invention;
b) introducing individual representatives of this library of chimeric DNA constructs in cells of the eukaryotic non-human organism, preferably by stable integration in their genome, particularly their nuclear genome;
c) isolating a eukaryotic organism exhibiting the particular trait; and
d) isolating the corresponding nucleic acid molecule present in the eukaryotic organism with the trait of interest, preferably from the aforementioned library.

It goes without saying that the methods and means of the invention may be used to determine the function of an isolated nucleic acid fragment or sequence with unknown function, by converting a part or the whole of that nucleic acid fragment or sequence according to the methods of the invention into a chimeric construct capable of making a dsRNA transcript when introduced in a eukaryotic cell, introducing that chimeric DNA construct into a eukaryotic organism to isolate preferably a number of transgenic organisms and observing changes in phenotypic traits.

The invention also provides acceptor vectors, as described in this specification as well as kits comprising the such vectors.

It goes without saying that the vectors, methods and kits according to the invention may be used in all eukaryotic organisms which are prone to gene silencing including yeast, fungi, plants, animals such as nematodes, insects and arthropods, vertebrates including mammals and humans.

Also provided by the invention are non-human organisms comprising chimeric DNA constructs comprising in sequence the following operably linked DNA fragments

i) a promoter or promoter region (3) capable of being recognized by RNA polymerases of the eukaryotic cell;
ii) a recombination site (15) which is the recombination product of the first (4) recombination site on the acceptor vector and the fifth recombination site (13) flanking the DNA of interest;

iii) a first DNA copy of the nucleic acid fragment of interest (12);

iv) a recombination site (16) which is the recombination product of the second (4) recombination site on the acceptor vector and the sixth recombination site (14) flanking the DNA of interest;

v) a recombination site (17) which is the recombination product of the third (5) recombination site on the acceptor vector and the sixth recombination site (14) flanking the DNA of interest;

vi) a second DNA copy of the nucleic acid fragment of interest in opposite orientation (12) compared to the first copy;

vii) a recombination site (18) which is the recombination product of the fourth (7) recombination site on the acceptor vector and the fifth recombination site (13) flanking the DNA of interest; and

viii) a 3' transcription terminating and polyadenylation region (8) functional in a eukaryotic cell.

As used herein “comprising” is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region which is functionally or structurally defined, may comprise additional DNA regions etc.

The term "gene" means any DNA fragment comprising a DNA region (the "transcribed DNA region") that is transcribed into a RNA molecule (e.g., a mRNA) in a cell operably linked to suitable regulatory regions, e.g., a plant-expressible promoter. A gene may thus comprise several operably linked DNA fragments such as a promoter, a 5' leader sequence, a coding region, and a 3' region comprising a polyadenylation site. A plant gene endogenous to a particular plant species (endogenous plant gene) is a gene which is naturally found in that plant species or which can be introduced in that plant species by conventional breeding. A chimeric gene is any gene which is not normally found in a plant species or, alternatively, any gene in which the

promoter is not associated in nature with part or all of the transcribed DNA region or with at least one other regulatory region of the gene.

The term "expression of a gene" refers to the process wherein a DNA region which is operably linked to appropriate regulatory regions, particularly to a promoter, is transcribed into an RNA which is biologically active i.e., which is either capable of interaction with another nucleic acid or which is capable of being translated into a polypeptide or protein. A gene is the to encode an RNA when the end product of the expression of the gene is biologically active RNA, such as e.g. an antisense RNA, a ribozyme or a replicative intermediate. A gene is the to encode a protein when the end product of the expression of the gene is a protein or polypeptide.

A nucleic acid is "capable of being expressed", when the nucleic acid, when introduced in a suitable host cell, particularly in a plant cell, can be transcribed (or replicated) to yield an RNA, and/or translated to yield a polypeptide or protein in that host cell.

The following non-limiting Examples describe the construction of acceptor vectors and the application thereof for the conversion of nucleic acid fragments of interest into chimeric DNA constructs capable of expressing a dsRNA transcript in eukaryotic cells. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology, Current Protocols*, USA. Standard materials and methods for plant molecular work are described in *Plant Molecular Biology Labfax* (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Other references for standard molecular biology techniques include Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual,* Third Edition, Cold Spring Harbor Laboratory Press, NY, Volumes I and II of Brown (1998) *Molecular Biology LabFax*, Second Edition, Academic Press (UK). Standard materials and methods for polymerase chain reactions can be found in Dieffenbach and Dveksler (1995) *PCR Primer: A Laboratory Manual*, Cold Spring

Harbor Laboratory Press, and in McPherson at al. (2000) *PCR - Basics: From Background to Bench*, First Edition, Springer Verlag, Germany.

Throughout the description and Examples, reference is made to the following sequences:

SEQ ID No 1:	core sequence of recombination site *att*B1
SEQ ID No 2:	core sequence of recombination site *att*B2
SEQ ID No 3:	core sequence of recombination site *att*B3
SEQ ID No 4:	core sequence of recombination site *att*R1
SEQ ID No 5:	core sequence of recombination site *att*R2
SEQ ID No 6:	core sequence of recombination site *att*R3
SEQ ID No 7:	core sequence of recombination site *att*L1
SEQ ID No 8:	core sequence of recombination site *att*L2
SEQ ID No 9:	core sequence of recombination site *att*L3
SEQ ID No 10:	core sequence of recombination site *att*P1
SEQ ID No 11:	core sequence of recombination sites *att*P2,P3
SEQ ID No 12:	nucleotide sequence of chalcone synthase gene of Arabidopsis
SEQ ID No 13:	nucleotide sequence of the acceptor vector "pHELLSGATE"
SEQ ID No 14:	oligonucleotide attB1 "forward" primer used for amplification of 400bp and 200 bp CHS fragments.
SEQ ID No 15:	oligonucleotide attB2 "reverse" primer for amplification of the 400 bp CHS fragment.
SEQ ID No 16:	oligonucleotide attB2 "reverse" primer for amplification of the 200 bp CHS fragment.
SEQ ID No 17:	oligonucleotide attB1 "forward" primer used for amplification of 100 bp CHS fragment.
SEQ ID No 18:	oligonucleotide attB2 "reverse" primer for amplification of the 100 bp CHS fragment.
SEQ ID No 19:	oligonucleotide attB1 "forward" primer used for amplification of 50 bp CHS fragment.
SEQ ID No 20:	oligonucleotide attB2 "reverse" primer for amplification of the 50 bp CHS fragment.

SEQ ID No 21:	oligonucleotide attB1 "forward" primer for amplification of the 25 bp CHS fragment.
SEQ ID No 22:	oligonucleotide attB2 "reverse" primer for the 25 bp fragment.
SEQ ID No 23:	nucleotide sequence of the acceptor vector "pHELLSGATE 4"
SEQ ID No 24:	nucleotide sequence of the acceptor vector "pHELLSGATE 8"
SEQ ID No 25:	nucleotide sequence of the acceptor vector "pHELLSGATE 11"
SEQ ID No 26:	nucleotide sequence of the acceptor vector "pHELLSGATE 12"

Examples

Example 1

Construction of the acceptor vector pHELLSGATE

With the completion of the *Arabidopsis* genome project, the advent of micro-array technology and the ever-increasing investigation into plant metabolic, perception, and response pathways, a rapid targeted way of silencing genes would be of major assistance. The high incidence and degree of silencing in plants transformed with chimeric genes containing simultaneously a sense and antisense nucleotide sequence, as well as a functional intron sequence suggested that such vectors could form the basis of a high-throughput silencing vector. However, one of the major obstacles in using such conventional cloning vectors for a large number of defined genes or a library of undefined genes would be cloning the hairpin arm sequences for each gene in the correct orientations.

Attempts to clone PCR products of sense and antisense arms together with the appropriately cut vector as a single step four-fragment ligation failed to give efficient or reproducible results. Therefore a construct (pHELLSGATE) was made to take advantage of Gateway™ (Life Technologies). With this technology, a PCR fragment is generated, bordered with recombination sites (*att*B1 and *att*B2) which is directionally recombined, *in vitro*, into a plasmid containing two sets of suitable recombination

sites (*att*P1 and *att*P2 sites) using the commercially available recombination protein preparation.

The pHELLSGATE vector was designed such that a single PCR product from primers with the appropriate *att*B1 and *att*B2 sites would be recombined into it simultaneously to form the two arms of the hairpin. The *ccd*B gene, which is lethal in standard *E.coli* strains such as DH5α (but not in DB3.1), was placed in the locations to be replaced by the arm sequences, ensuring that only recombinants containing both arms would be recovered. Placing a chloramphenicol resistance gene within the intron, gives a selection to ensure the retention of the intron in the recombinant plasmid.

pHELLSGATE comprises the following DNA fragments:

- a spectinomycin/streptomycin resistance gene(SEQ ID No 13 from the nucleotide at position 7922 to the nucleotide sequence at 9985);
- a right T-DNA border sequence (SEQ ID No 13 from the nucleotide at position 10706 to the nucleotide sequence at 11324);
- a CaMV35S promoter (SEQ ID No 13 from the nucleotide at position 11674 to the nucleotide sequence at 13019);
- an *att*P1 recombination site (complement of the nucleotide sequence of SEQ ID No 13 from the nucleotide at position 17659 to the nucleotide sequence at 17890);
- a *ccd*B selection marker (complement of the nucleotide sequence of SEQ ID No 13 from the nucleotide at position 16855 to the nucleotide at position 17610)
- an *att*P2 recombination site (complement of the nucleotide sequence of SEQ ID No 13 from the nucleotide at position 16319 to the nucleotide at position 16551)
- *pdk* intron2 (SEQ ID No 13 from the nucleotide at position 14660 to the nucleotide at position 16258) flanked by the intron splice site (TACAG*TT (SEQ ID No 13 from the nucleotide at position 16254 to the nucleotide sequence at 16260) and the intron splice site (TG*GTAAG) (SEQ ID No 13 from the nucleotide at position 14660 to the nucleotide sequence at 14667) and comprising a chloramphenicol resistance gene (SEQ ID No 13 from the nucleotide at position 15002 to the nucleotide at position 15661);

- an *att*P2 recombination site (SEQ ID No 13 from the nucleotide at position 14387 to the nucleotide at position 14619)
- a *ccd*B selection marker (complement of the nucleotide sequence of SEQ ID No 13 from the nucleotide at position 13675 to the nucleotide at position 13980)
- an *att*P1 recombination site (SEQ ID No 13 from the nucleotide at position 13048 to the nucleotide at position 13279)
- a octopine synthase gene terminator region (SEQ ID No 13 from the nucleotide at position 17922 to the nucleotide sequence at 18687);
- a chimeric marker selectable in plants comprising:
  - a nopaline synthase promoter (SEQ ID No 13 from the nucleotide at position 264 to the nucleotide sequence at 496);
  - a nptII coding region (SEQ ID No 13 from the nucleotide at position 497 to the nucleotide sequence at 1442); and
  - a nopaline synthase gene terminator (SEQ ID No 13 from the nucleotide at position 1443 to the nucleotide sequence at 2148);
- a left T-DNA border sequence (SEQ ID No 13 from the nucleotide at position 2149 to the nucleotide sequence at 2706);
- an origin of replication
- a kanamycin resistance gene

The complete nucleotide sequence of pHELLSGATE is represented in the sequence listing (SEQ ID No 13) and a schematic figure can be found in Figure 3.

Example 2

Use of the pHELLSGATE to convert nucleic acid fragments of interest into dsRNA producing chimeric silencing genes.

To test the acceptor vector pHELLSGATE an about 400bp, 200bp, 100bp, 50 bp and 25 bp fragment of the Arabidopsis thaliana chalcone synthase isomerase coding sequence (Seq ID No 12) (having respectively the nucleotide sequence of SEQ ID No 12 from the nucleotide at position 83 to the nucleotide at position 482; the nucleotide sequence of SEQ ID No 12 from the nucleotide at position 83 to the nucleotide at position 222; the nucleotide sequence of SEQ ID No 12 from the nucleotide at position 83 to the nucleotide at position 182; the nucleotide sequence of SEQ ID No 12 from

the nucleotide at position 83 to the nucleotide at position 132 ; and the nucleotide sequence of SEQ ID No 12 from the nucleotide at position 83 to the nucleotide at position 107) were used as nucleic acid fragments of insert for construction of chimeric genes capable of producing dsRNA.

This gene was chosen because its mutant allele has been reported in Arabidopsis to give distinct phenotypes. The CHS tt4(85) EMS mutant (Koornneef, 1990) produces inactive CHS resulting in no anthocyanin pigment in either the stem or seed-coat. Wildtype plants produce the purple-red pigment in both tissues.

In a first step, the respective fragments were PCR amplified using specific primers further comprising *att*B1 and *att*B2 recombination sites. *Att*B1 and *att*B2 specific primers were purchased from Life Technologies. The 25 and 50 bp fragments flanked by att sites were made by dimerization of the primers.

The following combinations of primers were used :

For the 400 bp fragment

Forward primer:

GGGGACAAGTTTGTACAAAAAAGCAGGCTGCACTGCTAACCCTGAGAACCATGTGCTTC (SEQ ID No 14); and

Reverse primer:

GGGGACCACTTTGTACAAGAAAGCTGGGTCGCTTGACGGAAGGACGGAGACCAAGAAGC (SEQ ID No 15).

For the 200 bp fragment

Forward primer:

GGGGACAAGTTTGTACAAAAAAGCAGGCTGCACTGCTAACCCTGAGAACCATGTGCTTC (SEQ ID No 14); and

Reverse primer:

GGGGACCACTTTGTACAAGAAAGCTGGGTAGGAGCCATGTAAGCACACATGTGTGGGTT (SEQ ID No 16).

For the 100 bp fragment

Forward primer:

GGGGACAAGTTTGTACAAAAAAGCAGGCTGCACTGCTAACCCTGAGAACCATGTG
CTTCAGGCGGAGTATCCTGACTACTACTTCCGCATCACCAACAGT (SEQ ID No 17); and

Reverse primer:

GGGGACCACTTTGTACAAGAAAGCTGGGTAACTTCTCCTTGAGGTCGGTCATGTG
TTCACTGTTGGTGATGCGGAAGTAGTAGTCAGGATACTCCGCCTG (SEQ ID No 18).

For the 50 bp fragment

Forward primer:

GGGGACAAGTTTGTACAAAAAAGCAGGCTGCACTGCTAACCCTGAGAACCATGTG
CTTCAGGCGGAGTATCCTGACTAC (SEQ ID No 19); and

Reverse primer:

GGGGACCACTTTGTACAAGAAAGCTGGGTGTAGTCAGGATACTCCGCCTGAAGCA
CATGGTTCTCAGGGTTAGCAGTGC (SEQ ID No 20).

For the 25 bp fragment

Forward primer:

GGGGACAAGTTTGTACAAAAAAGCAGGCTGCACTGCTAACCCTGAGAACCATGT (SEQ ID No 21); and

Reverse primer:

GGGGACCACTTTGTACAAGAAAGCTGGGTACATGGTTCTCAGGGTTAGCAGTGC (SEQ ID No 22).

PCR amplification and recombination using the GATEWAY™ technology with the commercially available BP Clonase (Life Technologies) were performed according to the manufacturer's instructions (manual available on http://www.lifetech.com/content.cfm?pageid=2497).

Bacterial colonies obtained on chloramphenicol-containing plates spread with *E. coli* DH5α bacteria, transformed (by electroporation or by heatshocking RbCl2 treated competent *E. coli* cells) with the *in vitro* recombination reaction were screened. Colonies containing the desired recombinant plasmid were obtained in each case. For the about 400 bp fragment 24 colonies were screened and 23 contained the desired construct with the 400 bp in inverted repeat, operably linked to the CaMV35S promoter. For the about 200 bp fragment 36 colonies were screened and 35 contained the desired construct with the 200 bp in inverted repeat, operably linked to the CaMV35S promoter. For the about 50 bp fragment 6 colonies were screened and 4 contained the desired construct with the 50 bp in inverted repeat, operably linked to the CaMV35S promoter. For the 25 bp fragment, 6 colonies were screened and 1 contained the desired construct with the 400 bp in inverted repeat, operably linked to the CaMV35S promoter. In a number of cases the structure was confirmed by sequence analysis.

These results show that this vector facilitates the rapid, efficient, and simple production of hpRNA (hairpin RNA constructs). pHELLSGATE is a T-DNA vector, with a high-copy-number origin of replication for ease of handling. Recombinant pHELLSGATE constructs can be directly transformed into *Agrobacterium* for transformation of the chimeric construct into plants. This system can be used in high throughput applications.

Example 3

Evaluation of plants comprising the chimeric genes of Example 2.

The vectors containing the dsRNA producing chimeric constructs with the 400, 200, 100, 50 and 25 nucleotides of chalcone synthase in inverted repeat (Example 2) were introduced into *Agrobacterium tumefaciens* strain AGL1, GV3101 or LBA4404 either by electroporation or tri-parental mating.

Transgenic Arabidopsis lines are obtained by transformation with these Agrobacteria using the dipping method of Clough and Bent (1998).

Chalcone synthase activity is monitored by visual observation of stem and leaf color (normally in plants grown under high light, and by unaided or microscope assisted visual observation of seed-coat color.

Most of the transgenic lines transformed with the above mentioned CHS silencing constructs show pronounced silencing. The seed colour of most of these lines is virtually indistinguishable from seed of the tt4(85) mutant to the naked eye . Examination of the seed under a light microscope reveals that the degree of pigmentation is generally uniform in the cells of the coat of an individual seed, and among seeds of the same line.

Example 4

Construction of the acceptor vectors pHELLSGATE 4, pHELLSGATE 8, pHELLSGATE 11 and pHELLSGATE 12.

pHELLSGATE 4 was made by excising the DNA fragment comprising the *pdk* intron and chloramphenicol resistance gene from pHELLSGATE (Example 1) with *Hin*dIII and *Eco*RI and replacing it with a *Hin*dIII/*Eco*RI DNA fragment containing only the *pdk* intron. The complete nucleotide sequence of pHELLSGATE 4 is represented in the sequence listing (SEQ ID No 23).

pHELLSGATE 8 was made by PCR amplification using pHellsgate DNA as a template and oligonucleotides with the sequence 5'GGGCTCGAGACAAGTTTGTACAAAAAAGCTG 3' and 5'GGCTCGAGACCACTTTGTACAAGAAAGC 3' as primers. These primers modify the attP sites within pHellsgate to attR sites. The resulting fragment was sequenced and inserted into the *Xho*I site of a vector upstream of a DNA fragment containing the *pdk* intron fragment. Similarly an *XbaI*/XbaI fragment amplified with the oligonucleotides 5'GGGTCTAGACAAGTTTGTACAAAAAAGCTG 3' and 5' GGGTCTAGACCACTTTGTACAAGAAAGC 3' as primers and pHEllSGATE as template DNA to modify the attP sites of this cassette to attR sites. This fragment was sequenced and inserted into the *Xba*I site of the intermediate described above downstream of the *pdk* intron. The complete nucleotide sequence of pHELLSGATE 8 is represented in the sequence listing (SEQ ID No 24) and a schematic figure can be found in Figure 4.

pHELLSGATE 11 is similar to pHELLSGATE 8 except that the pdk intron has been engineered to contain a branching point in the complementary strand such that splicing of the intron is independent of its orientation (a so-called "two-way intron"). The complete nucleotide sequence of pHELLSGATE 11 is represented in the sequence listing (SEQ ID No 25) and a schematic representation thereof can be found in Figure 4.

pHELLSGATE 12 is also similar to pHELLSGATE 8 except that the pdk intron has been duplicated as an inverted repeat. The complete nucleotide sequence of pHELLSGATE 12 is represented in the sequence listing (SEQ ID No 26) and a schematic representation thereof can be found in Figure 4.

Example 5

Use of the different pHELLSGATE vectors to generate dsRNA chimeric silencing genes targeted towards three different model target genes.

The efficiency in gene silencing of the different pHELLSGATE vectors was tested by inserting fragments of three target genes Flowering locus C (FLC) Ethylene insensitive 2 (EIN2) and Phytoene desaturase (PDC). For FLC a 390 bp fragment was used (from the nucleotide at position 303 to the nucleotide at position 692 of the nucleotide sequence available as Genbank Accession Nr AF116527) . For EIN2 a 580 bp fragment was used (from the nucleotide at position 541 to the nucleotide at position 1120 of the nucleotide sequence available as Genbank Accession Nr AF141203). For PDS a 432 bp fragment was used (from the nucleotide at position 1027 to the nucleotide at position 1458 of the nucleotide sequence available as Genbank Accession Nr L16237). Genes of interest were amplified using gene specific primers with either a 5' attB1 extension (GGGGACAAGTTTGTACAAAAAAGCAGGCT) or an attB2 extension (GGGACCACTTTGTACAAGAAAGCTGGGT) using F1 Taq DNA polymerase (Fisher Biotec, Subiaco, WA, Australia) according to the manufacturer's protocol. PCR products were precipitated by adding 3 volumes TE and two volumes 30% (w/v) PEG 3000, 30mM $MgCl_2$ and centrifuging at 13000 g for 15 minutes. Recombination reaction of PCR products with either pDONR201 (Invitrogen, Groningen, The

Netherlands) or pHELLSGATE 4 were carried out in a total volume of 10 μL with 2 μL BP clonase buffer (Invitrogen), 1-2 μL PCR product 150 ng plasmid vector and 2 μL BP clonase (Invitrogen). The reaction was incubated at room temperature (25°C) for 1 h to overnight. After the incubation, 1 μL proteinase K (2 μg/μL; Invitrogen) was added and incubated for 10 min at 37°C. 1-2 μL of the mix was used to transform DH5α, colonies were selected on the appropriate antibiotics. Clones were checked either by digestion of DNA minipreps or PCR. Recombination reactions from pDONR201 clones to pHellsgate 8, 11 or 12 were carried out in 10 μL total volume with 2 μL LR clonase buffer (Invitrogen), 2 μL pDONR201 clone (approximately 150 ng), 300 ng pHellsgate 8, 11 or 12 and 2 μL LR clonase (Invitrogen). The reaction was incubated overnight at room temperature, proteinase-treated and used to transform E. coli DH5α as for the BP clonase reaction. Transformation of Arabidopsis was perfomed according to via the floral dip method (Clough and Bent, 1998). Plants were selected on agar solidified MS media supplemented with 100 mg/l timentin and 50 mg/l kanamycin. For *FLC* and *PDS* constructs the C24 ecotype was used; for *EIN2* constructs Landsberg *erecta* was used. For scoring of *EIN2* phenotypes transformed T1 plants were transferred to MS media containing 50 μM 1-aminocyclopropane-1-carboxylic acid (ACC) together with homozygous *EIN2*-silenced lines and wild type Landberg erecta plants. T1 *FLC* hpRNA plants were scored by transferring to MS plates and scoring days to flower or rosette leaves at flowering compared to C24 wild type plants and *flc* mutant lines. T1 PDS hpRNA plants were scored by looking at bleaching of the leaves. The results of the analysis of plants transformed with the different pHELLSGATE vectors are shown in Table 1.

All plants transformed with pHellsgate 4-FLC and pHellsgate 8-FLC flowered significantly earlier than wildtype C24 and in both cases plants flowering with the same number of rosette leaves as the *flc-20* line (carrying a stable Ds insertion in the first intron of the *FLC* gene) were observed. There was no clear difference in rosette leaves at flowering between the sets of plants transformed with the pHELLSGATE 4-FLC and pHellsgate 8-FLC constructs.

A difference in the effectiveness of the pHELLSGATE 4-EIN2 and pHELLSGATE 8-EIN2 plants was observed. Of 36 transformants for pHG4-EIN2 there were no plants

with an observable ACC-resistant phenotype under the conditions used for this experiment, whereas 8 of the 11 plants carrying the pHG8-EIN2 transgene showed some degree of ACC-resistance. The extent to which the pHG8-EIN2 plants were resistant to ACC was variable indicating that the severity of silencing varies between transformants.

The great majority of plants carrying pHG4-PDS and pHG8-PDS showed a phenotype consistent with the loss of photoprotection due to the absence of carotenoids. The weakest phenotype was a bleaching of the cotyledons, with the true leaves not bleaching at any stage in the life cycle. The bleached cotyledon phenotype was only seen in plants transformed with *PDS* hpRNA constructs; we confirmed that the plants with this phenotype also contained the *PDS* hpRNA construct (data not shown) strongly suggesting that this phenotype is due to *PDS* silencing and not bleaching from the kanamycin selection. Plants transformed with the pHELLSGATE 4-PDS construct gave only this weak bleached cotyledon phenotype. In contrast the five of the pHELLSGATE 8-PDS plants had the weak phenotype and three showed a stronger phenotype with extensive or complete bleaching of the true leaves.

Table 1

Construct	Test genes	T1 plants	Rate of silencing
HELLSGATE 4	FLC EIN2 PDS	13 36 12	12 0 11
HELLSGATE 8	FLC EIN2 PDS	6 11 9	6 8 8
HELLSGATE 11	FLC EIN2 PDS	2 30 11	2 11 11
HELLSGATE 11 (intervening region in inverse orientation)	FLC EIN2 PDS	8	6
HELLSGATE 12	FLC EIN2 PDS	13 26	11 12
HELLSGATE 12 (intervening region in inverse orientation)	FLC EIN2 PDS CHS	9 5	8 2

References

An *et al.* (1996) *The Plant Cell* 8, 15-30

AzpiroLeehan and Feldmann (1997) *Trends Genet.* 13: 152-156

Clough and Bent (1998) *Plant J.* 16: 735-743

Fire *et al.* (1998) *Nature* 391: 806-811

Fromm *et al.* (1990) *Bio/Technology* 8: 833

Gordon-Kamm *et al.* (1990) *The Plant Cell* 2: 603

Hamilton *et al.* (1998) *Plant J.* 15: 737-746

Hoess *et al.* (1986) *Nucl. Acids Res.* 14: 2287

Hudspeth *et al.* (1989) *Plant Mol. Biol.* 12: 579-589

Keil *et al.* (1989) *EMBO J.* 8: 1323-1330

Keller *et al.* (1988) *EMBO J.* 7: 3625-3633

Keller *et al.* (1989) *Genes & Devel.* 3: 1639-1646

Koornneef (1990) *Theor. Appl. Gen.* 80: 852-857

Landy (1993) *Current Opinions in Genetics and Development* 3: 699-707

Landy (1989) *Ann. Rev. Biochem.* 58: 913

Martienssen (1998) *Proc. Natl. Acad. Sci. USA* 95: 2021-2026

Meyer *et al.* (1987) *Nature* 330: 677

Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443-453

Peleman *et al.* (1989) *Gene* 85: 359-369

Ross-MacDonald *et al.* (1999) *Nature* 402: 413-418

Smith *et al.* (2000) *Nature* 407: 319-320

Wagner and Sun (1998) *Nature* 391: 744-745

Waterhouse *et al* (1998) *Proc. Natl. Acad. Sci. USA* 95: 13959-13964

Claims:

1. A vector comprising the following operably linked DNA fragments:
   a) an origin of replication allowing replication in a recipient cell (1), preferably in bacteria; particularly in *Escherichia coli*.
   b) a selectable marker region (2) capable of being expressed in said recipient cell; and
   c) a chimeric DNA construct comprising in sequence:
      i) a promoter or promoter region (3) capable of being recognized by RNA polymerases of a eukaryotic cell;
      ii) a first recombination site (4), a second recombination site (5), a third recombination site (6) and a fourth recombination site (7);
      iii) a 3' transcription terminating and polyadenylation region (8) functional in said eukaryotic cell;

   wherein said first recombination site (4) and said fourth recombination site (7) are capable of reacting with a same recombination site, preferably are identical, and said second recombination site (5) and said third recombination site (6), are capable of reacting with a same recombination site, preferably are identical; and wherein said first recombination site (4) and said second recombination site (5) do not recombine with each other or with a same recombination site or said third recombination site (6) and said fourth recombination site (7) do not recombine with each other or with a same recombination site.

2. The vector of claim 1, wherein said first (4) and second recombination site (5) flank a second selectable marker gene (10) and said third (6) and fourth recombination site (7) flank a third selectable marker gene (9).

3. The vector of claim 1 or 2, wherein said chimeric DNA construct comprises a region flanked by intron processing signals (11), functional in said eukaryotic cell, located between said second recombination site (5) and said third recombination site (6).

4. The vector of claim 3, wherein said region flanked by intron processing signals is an intron sequence functional in said eukaryotic cell.

5. The vector of any one of claims 3 or 4, further comprising a fourth selectable marker gene (19), located between said second (5) and third recombination site (6).

6. The vector of any one of claims 1 to 5, wherein said selectable marker genes are selected from the group consisting of an antibiotic resistance gene, a tRNA gene, an auxotrophic marker, a toxic gene, a phenotypic marker, an antisense oligonucleotide; a restriction endonuclease; a restriction endonuclease cleavage site, an enzyme cleavage site, a protein binding site, an a sequence complementary PCR primer.

7. The vector of any one of claims 1 to 6, wherein said promoter (3) is a plant-expressible promoter.

8. The vector of any one of claim 7, wherein said chimeric DNA construct is flanked by left and right border T-DNA sequences.

9. The vector of claim 8, further comprising a selectable marker gene capable of being expressed in plant cells located between said left and said right T-DNA border sequences.

10. The vector of claim 8 or claim 9, further comprising an origin of replication capable of functioning in Agrobacterium sp.

11. The vector of any one of claims 1 to 10, wherein said first (4) and fourth recombination site (7) is attR1 comprising the nucleotide sequence of SEQ ID No 4 and said second (5) and third (6) recombination site is attR2 comprising the nucleotide sequence of SEQ ID No 5.

12. The vector of any one of claims 1 to 10, wherein said first (4) and fourth recombination site (7) is attP1 comprising the nucleotide sequence of SEQ ID No 10 and said second (5) and third (6) recombination site is attP2 comprising the nucleotide sequence of SEQ ID No 11.

13. A vector comprising the sequence of SEQ ID No 13.

14. A vector comprising the sequence of SEQ ID No 23.

15. A vector comprising the sequence of SEQ ID No 24.

16. A vector comprising the sequence of SEQ ID No 25.

17. A vector comprising the sequence of SEQ ID No 26.

18. A vector comprising the following operably linked DNA fragments:
   a) an origin of replication allowing replication in a recipient cell (1), preferably in bacteria; particularly in *Escherichia coli*.
   b) a selectable marker region (2) capable of being expressed in said recipient cell; and
   c) a chimeric DNA construct comprising in sequence:
      i) a promoter or promoter region (3) capable of being recognized by a prokaryotic RNA polymerase;
      ii) a first recombination site (4), a second recombination site (5), a third recombination site (6) and a fourth recombination site (7);
      iii) a 3' transcription terminating and polyadenylation region (8) functional in said eukaryotic cell;

wherein said first recombination site (4) and said fourth recombination site (7) are capable of reacting with a same recombination site, preferably are identical, and said second recombination site (5) and said third recombination site (6), are capable of reacting with a same recombination site, preferably are identical; and wherein said first recombination site (4) and said second recombination site (5) do not recombine with each other or with a same recombination site or said third recombination site (6) and said fourth recombination site (7) do not recombine with each other or with a same recombination site.

19. The vector of claim 18, wherein said RNA polymerase is a bacteriophage single subunit RNA polymerase.

20. A kit comprising the vector according to any one of claims 1 to 19.

21. The kit of claim 20, further comprising at least one recombination protein capable of recombining a DNA segment comprising at least one of said recombination sites.

22. A method for making a chimeric DNA construct capable of expressing a dsRNA in a eukaryotic cell comprising the step of
    a) combining in vitro:
        i) a vector according to any one of claims 1 to 19;
        ii) an insert DNA comprising a DNA segment of interest (12) flanked by
            (1) a fifth recombination site (13) which is capable of recombining with said first (4) or fourth recombination site (7) on said vector; and
            (2) a sixth recombination site (14) which is capable of recombining with said second (5) or third recombination site (6) on said vector;
        iii) at least one site specific recombination protein capable of recombining said first (4) or fourth (7) and said fifth recombination site (13) and said second (5) or third (6) and said sixth recombination site (14);
    b) allowing recombination to occur so as to produce a reaction mixture comprising product DNA molecules, said product DNA molecule comprising in sequence:
        i) said promoter or promoter region (3) capable of being recognized by RNA polymerases of said eukaryotic cell;
        ii) a recombination site (15) which is the recombination product of said first (4) and said fifth recombination site (13);
        iii) said DNA fragment of interest (12);
        iv) a recombination site (16) which is the recombination product of said second (4) and said sixth recombination site (14);
        v) a recombination site (17) which is the recombination product of said third (5) and said sixth recombination site (14);
        vi) said DNA fragment of interest in opposite orientation (12);

vii) a recombination site (18) which is the recombination product of said fourth (7) and said fifth recombination site (13); and

viii) said 3' transcription terminating and polyadenylation region (8) functional in said eukaryotic cell;

c) selecting said product DNA molecules.

23. The method according to claim 22, wherein said selecting is carried out in vivo.

24. The method according to claim 22 or 23, wherein said insert DNA is a linear DNA molecule.

25. The method according to claim 22 or 23, wherein said insert DNA is a circular DNA molecule.

26. The method according to any of claims 22 to 25, wherein said at least one recombination protein is selected from (i) Int and IHF and (ii) Int, Xis, and IHF.

27. The method according to any one of claims 22 to 25, wherein multiple insert DNAs comprising different DNA fragments of interest are processed simultaneously.

28. A method for preparing a eukaryotic non-human organism wherein the phenotypic expression of a target nucleic acid of interest is reduced or inhibited, said method comprising:

a) preparing a chimeric DNA construct comprising a nucleic acid of interest (12) comprising a nucleotide sequence of at least 19 bp with at least 70% sequence identity to said target nucleic acid capable of expressing a dsRNA in cells of said eukaryotic non-human organism according to any one of the methods of claims 22 to 27;

b) introducing said chimeric DNA construct in cells of said eukaryotic non-human organism; and

c) isolating said eukaryotic organism

29. The method of claim 28, wherein said eukaryotic organism is a plant.

30. A method for isolating a nucleic acid molecule involved in determining a particular trait
    a) preparing a library of chimeric DNA constructs capable of expressing a dsRNA in cells of said eukaryotic non-human organism according to any one of the methods of claims 22 to 27;
    b) introducing individual representatives of said library of chimeric DNA constructs in cells of said eukaryotic non-human organism;
    c) isolating a eukaryotic organism exhibiting said particular trait; and
    d) isolating said nucleic acid molecule.

31. The method according to claim 30, wherein said eukaryotic organism is a plant.

32. A eukaryotic non-human organism comprising a chimeric DNA construct obtainable through the methods of any one of claims 22 to 27.

33. The non-human eukaryotic organism according to claim 31 which is a plant.

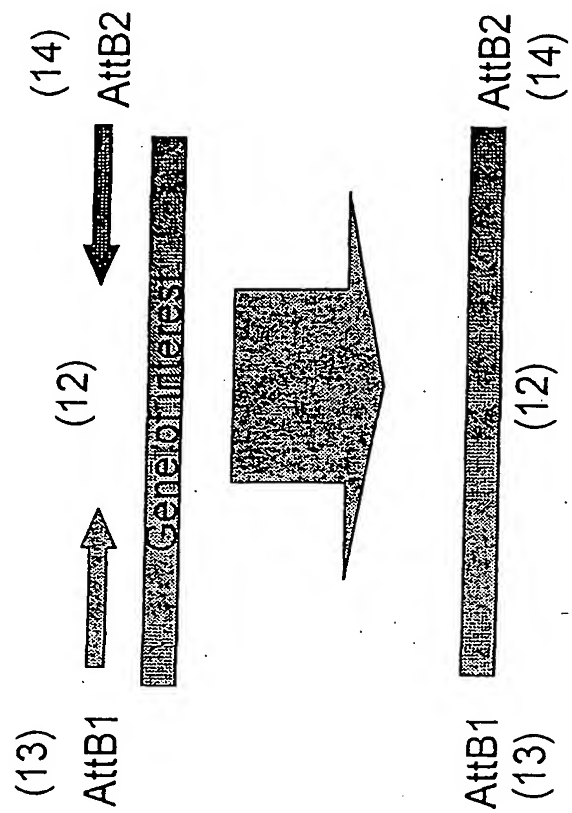


Figure 1A

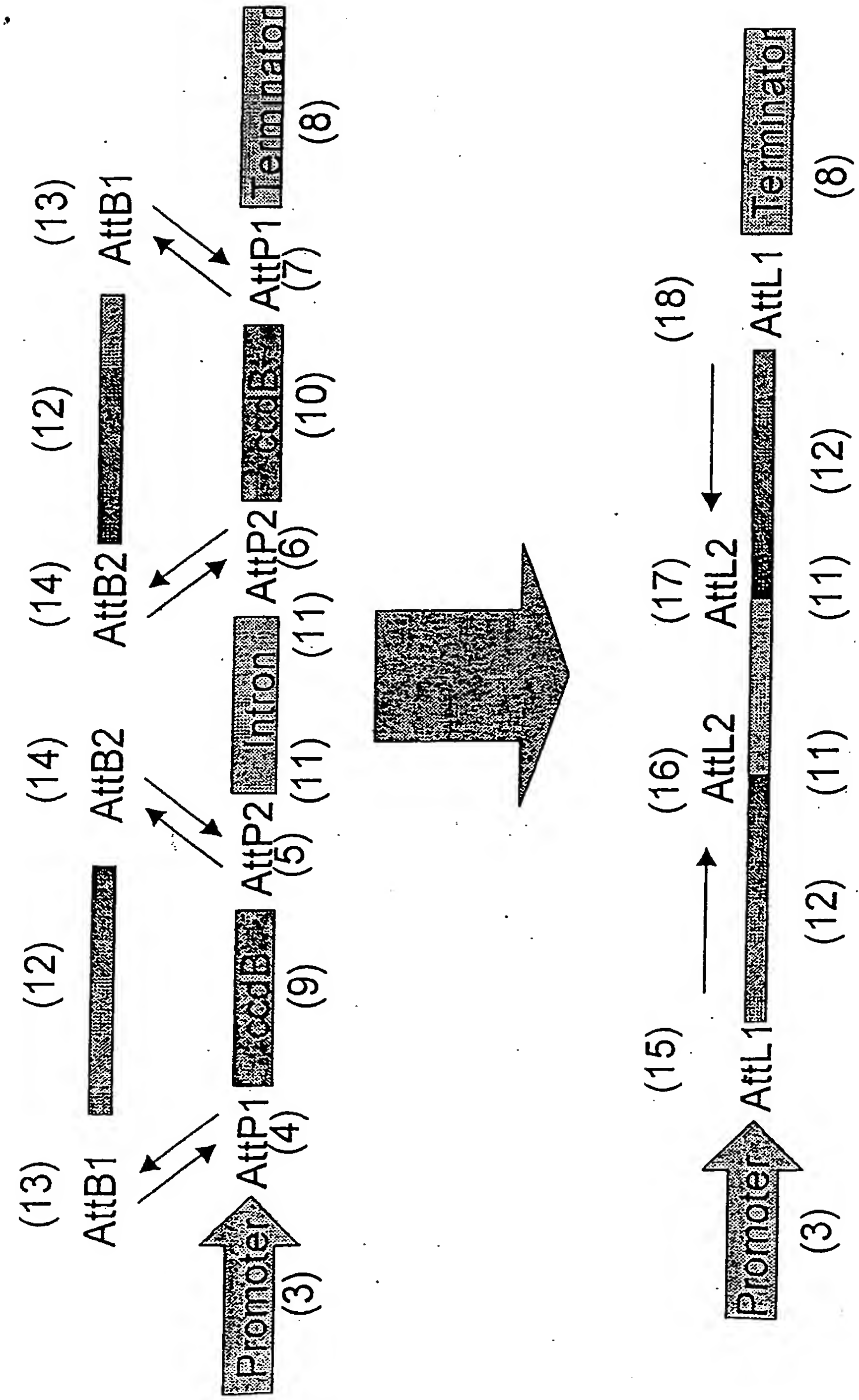

Promoter
(3)
AttB1
(13)
AttP1
(4)
(12)
ccdB
(9)
AttB2
(14)
AttP2
(5)
Intron
(11)
AttP2
(6)
ccdB
(10)
AttP1
(7)
Terminator
(8)
AttL1
(15)
AttL2
(16)
(17)
(18)

Figure 1B

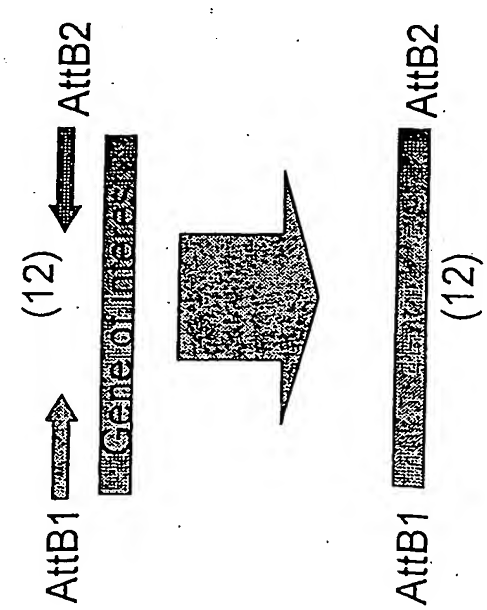


Figure 2A

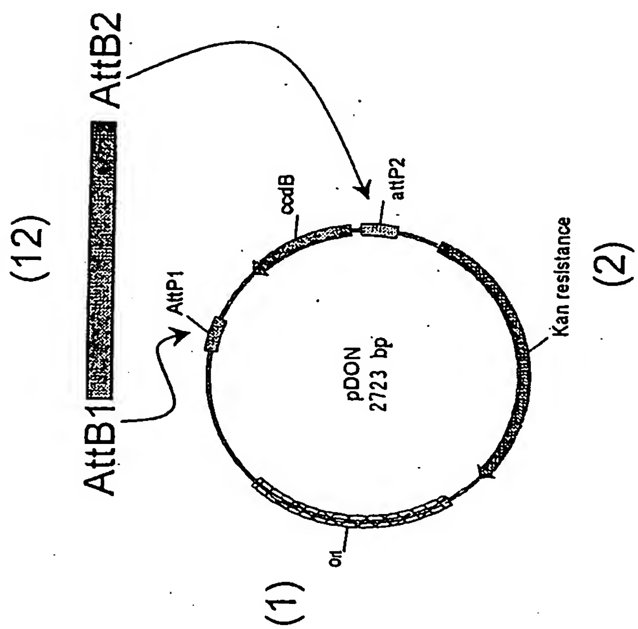


Figure 2B

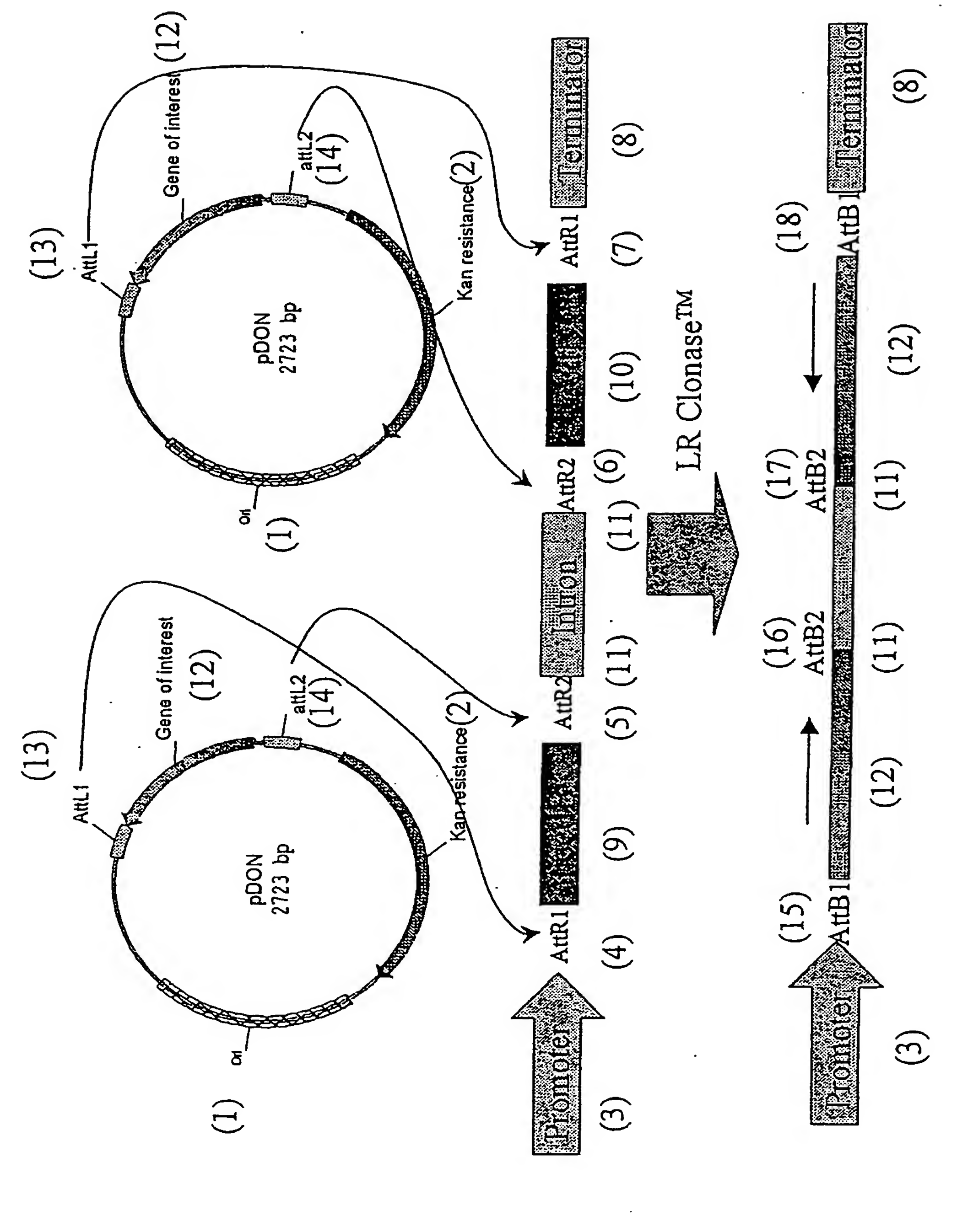

(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(11)
(12)
(13)
(14)
(15)
(16)
(17)
(18)
pDON
2723 bp
AttL1
attL2
Gene of interest
Kan resistance
Ori
Promoter
AttR1
AttR2
Intron
Terminator
LR Clonase™
AttB1
AttB2

Figure 2C

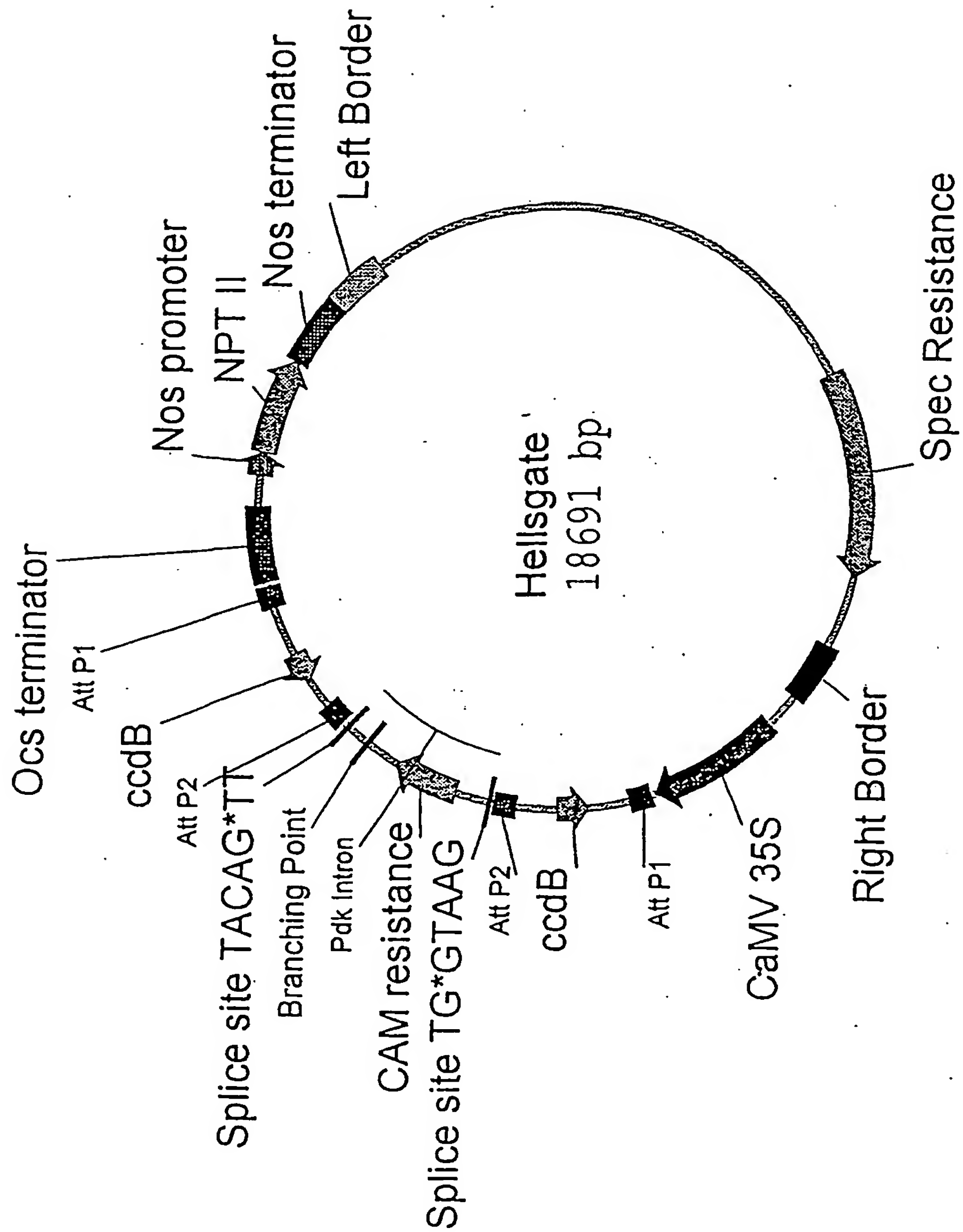

Hellsgate
18691 bp
Left Border
Nos terminator
NPT II
Nos promoter
Ocs terminator
Att P1
ccdB
Att P2
Splice site TACAG*TT
Branching Point
Pdk Intron
CAM resistance
Splice site TG*GTAAG
Att P2
ccdB
Att P1
CaMV 35S
Right Border
Spec Resistance

Figure 3

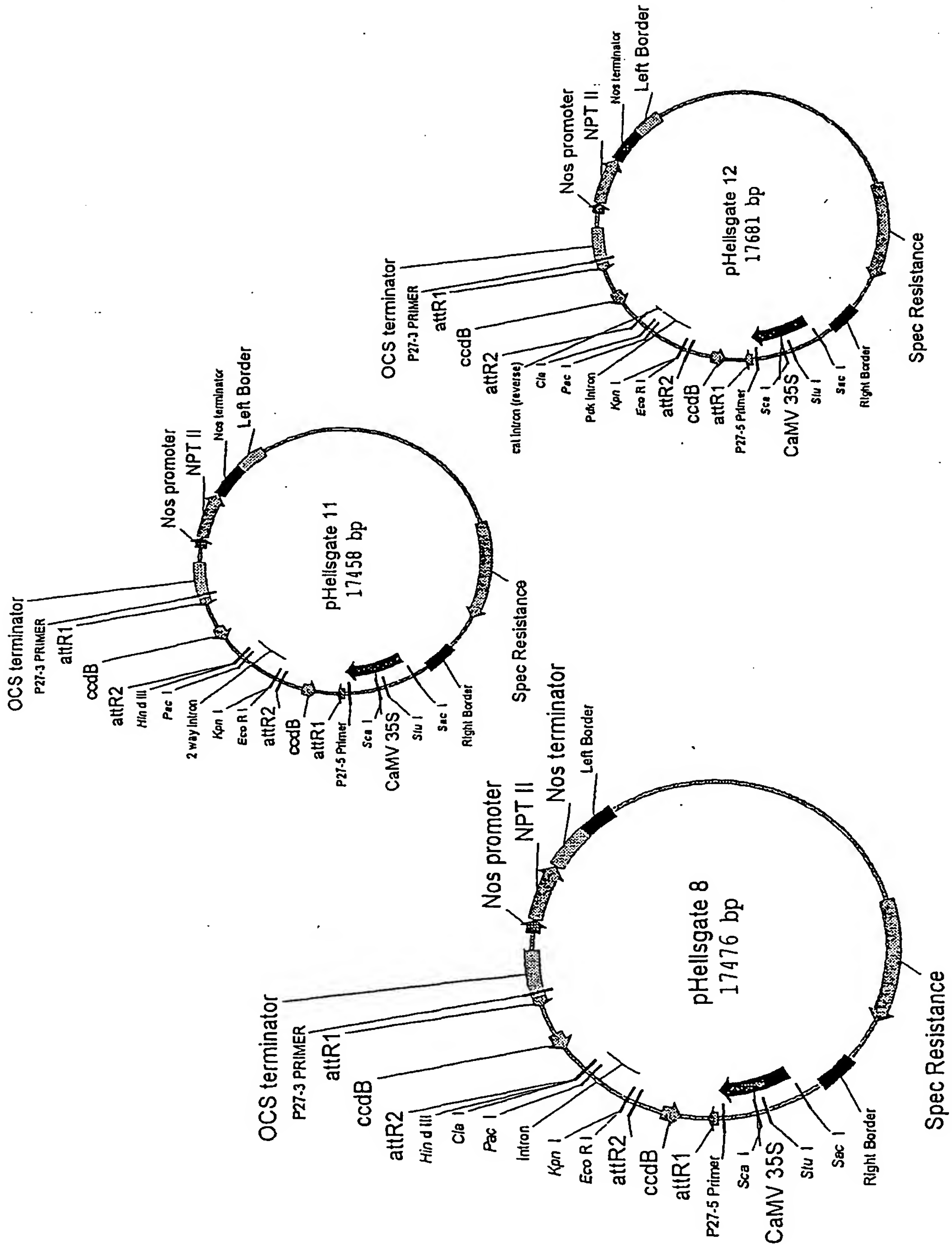

pHellsgate 12
17681 bp
Nos promoter
NPT II
Nos terminator
Left Border
OCS terminator
P27-3 PRIMER
attR1
ccdB
attR2
Cla I
Pac I
cat Intron (reverse)
Pdk Intron
Kpn I
Eco R I
attR2
ccdB
attR1
P27-5 Primer
Sca I
CaMV 35S
Stu I
Sac I
Right Border
Spec Resistance
pHellsgate 11
17458 bp
Nos promoter
NPT II
Nos terminator
Left Border
OCS terminator
P27-3 PRIMER
attR1
ccdB
attR2
Hin d III
Pac I
2 way Intron
Kpn I
Eco R I
attR2
ccdB
attR1
P27-5 Primer
Sca I
CaMV 35S
Stu I
Sac I
Right Border
Spec Resistance
pHellsgate 8
17476 bp
Nos promoter
NPT II
Nos terminator
Left Border
OCS terminator
P27-3 PRIMER
attR1
ccdB
attR2
Hin d III
Cla I
Pac I
Intron
Kpn I
Eco R I
attR2
ccdB
attR1
P27-5 Primer
Sca I
CaMV 35S
Stu I
Sac I
Right Border
Spec Resistance

Figure 4

SEQUENCE LISTING

<110> COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION

<120> Method and means for producing efficient silencing constructs using recombinational cloning

<130> 500255/MRO

<150> US60/264,067
<151> 2001-01-26

<150> US60/333,743
<151> 2001-11-29

<160> 26
<170> PatentIn version 3.1

<210> 1
<211> 25
<212> DNA
<213> Artificial sequence

<220>
<223> core sequence of recombination site attB1

<400> 1
agcctgcttt tttgtacaaa cttgt 25

<210> 2
<211> 25
<212> DNA
<213> Artificial sequence

<220>
<223> core sequence of recombination site attB2

<400> 2
agcctgcttt cttgtacaaa cttgt 25

<210> 3
<211> 25
<212> DNA
<213> Artificial sequence

<220>
<223> core seqeunce of recombination site attB3

<400> 3
acccagcttt cttgtacaaa cttgt 25

<210> 4
<211> 25
<212> DNA
<213> Artificial sequence

<220>
<223> core sequence of recombination site attR1

<400> 4
gttcagcttt tttgtacaaa cttgt 25

<210> 5
<211> 25
<212> DNA
<213> Artificial sequence

<220>
<223> core sequence of recombination site attR2

<400> 5
gttcagcttt cttgtacaaa cttgt 25

<210> 6
<211> 25
<212> DNA
<213> Artificial sequence

<220>
<223> core sequence of recombination site attR3

<400> 6
gttcagcttt cttgtacaaa gttgg 25

<210> 7
<211> 25
<212> DNA
<213> Artificial sequence

<220>
<223> core sequence of recombination site attL1

<400> 7
agcctgcttt tttgtacaaa gttgg 25

<210> 8
<211> 25
<212> DNA
<213> Artificial sequence

<220>
<223> core sequence of recombination site attL2

<400> 8
agcctgcttt cttgtacaaa gttgg 25

<210> 9
<211> 25
<212> DNA
<213> Artificial sequence

<220>

<223> core sequence of recombination site attL3

<400> 9
acccagcttt cttgtacaaa gttgg 25

<210> 10
<211> 25
<212> DNA
<213> Artificial sequence

<220>
<223> core sequence of recombination site attP1

<400> 10
gttcagcttt tttgtacaaa gttgg 25

<210> 11
<211> 25
<212> DNA
<213> Artificial sequence

<220>
<223> core sequence of recombination site attP2,P3

<400> 11
gttcagcttt cttgtacaaa gttgg 25

<210> 12
<211> 1188
<212> DNA
<213> Artificial sequence

<220>
<223> cDNA sequence of the Arabidopsis thaliana chalcone synthase codin
g region

<400> 12
atggtgatgg ctggtgcttc ttctttggat gagatcagac aggctcagag agctgatgga 60

cctgcaggca tcttggctat tggcactgct aaccctgaga accatgtgct tcaggcggag 120

tatcctgact actacttccg catcaccaac agtgaacaca tgaccgacct caaggagaag 180

ttcaagcgca tgtgcgacaa gtcgacaatt cggaaacgtc acatgcatct gacggaggaa 240

ttcctcaagg aaaacccaca catgtgtgct tacatggctc cttctctgga caccagacag 300

gacatcgtgg tggtcgaagt ccctaagcta ggcaaagaag cggcagtgaa ggccatcaag 360

gagtggggcc agcccaagtc aaagatcact catgtcgtct tctgcactac ctccggcgtc 420

gacatgcctg gtgctgacta ccagctcacc aagcttcttg gtctccgtcc ttccgtcaag 480

cgtctcatga tgtaccagca aggttgcttc gccggcggta ctgtcctccg tatcgctaag 540

gatctcgccg agaacaaccg tggagcacgt gtcctcgttg tctgctctga gatcacagcc 600

gttaccttcc gtggtccctc tgacacccac cttgactccc tcgtcggtca ggctcttttc 660

agtgatggcg ccgccgcact cattgtgggg tcggaccctg acacatctgt cggagagaaa 720

cccatctttg agatggtgtc tgccgctcag accatccttc cagactctga tggtgccata 780

gacggacatt tgagggaagt tggtctcacc ttccatctcc tcaaggatgt tcccggcctc 840

atctccaaga acattgtgaa gagtctagac gaagcgttta aacctttggg gataagtgac 900

tggaactccc tcttctggat agcccacccct ggaggtccag cgatcctaga ccaggtggag 960

ataaagctag gactaaagga agagaagatg agggcgacac gtcacgtgtt gagcgagtat 1020

ggaaacatgt cgagcgcgtg cgttctcttc atactagacg agatgaggag gaagtcagct 1080

aaggatggtg tggccacgac aggagaaggg ttggagtggg gtgtcttgtt tggtttcgga 1140

ccaggtctca ctgttgagac agtcgtcttg cacagcgttc ctctctaa 1188

<210> 13
<211> 18691
<212> DNA
<213> Artificial sequence

<220>
<223> acceptor vector pHELLSGATE

<220>
<221> misc_feature
<222> (7922)..(9985)
<223> spectinomycin resistance

<220>
<221> misc_feature
<222> (10706)..(11324)
<223> right T-DNA border fragment

<220>
<221> misc_feature
<222> (11674)..(13019)
<223> CaMV35S promoter fragment

<220>
<221> misc_feature
<222> (17890)..(17659)
<223> attP1 recombination site (complement)

<220>
<221> misc_feature
<222> (17610)..(16855)
<223> ccdB selection marker (complement)

<220>
<221> misc_feature
<222> (16551)..(16319)
<223> attP2 recombination site (complement)

<220>
<221> misc_feature
<222> (14660)..(16258)
<223> pdk2 intron 2

<220>
<221> misc_feature
<222> (15002)..(15661)
<223> chloramphenicol resistance gene

<220>
<221> misc_feature
<222> (14387)..(14619)
<223> attP2 recombination site

<220>
<221> misc_feature
<222> (13675)..(13980)
<223> ccdB selection marker (complement)

<220>
<221> misc_feature
<222> (13048)..(13279)
<223> attP1 recombination site

<220>
<221> misc_feature
<222> (17922)..(18687)
<223> octopine synthase gene terminator region

<220>
<221> misc_feature
<222> (264)..(496)
<223> nopaline synthase gene promoter

<220>
<221> misc_feature
<222> (497)..(1442)
<223> nptII coding region

<220>
<221> misc_feature
<222> (1443)..(2148)
<223> nopaline synthase gene terminator

<220>
<221> misc_feature
<222> (2149)..(2706)
<223> a left T-DNA border region

<400> 13

ggccgcacta gtgatatccc gcggccatgg cggccgggag catgcgacgt cgggcccaat 60
tcgccctata gtgagtcgta ttacaattca ctggccgtcg ttttacaacg tcgtgactgg 120
gaaaaccctg gcgttaccca acttaatcgc cttgcagcac atcccccttt cgccagctgg 180
cgtaatagcg aagaggcccg caccgatcgc ccttcccaac agttgcgcag cctgaatggc 240
gaatggaaat tgtaaacgtt aatgggtttc tggagtttaa tgagctaagc acatacgtca 300
gaaaccatta ttgcgcgttc aaaagtcgcc taaggtcact atcagctagc aaatatttct 360
tgtcaaaaat gctccactga cgttccataa attcccctcg gtatccaatt agagtctcat 420
attcactctc aatccaaata atctgcaatg gcaattacct tatccgcaac ttctttacct 480
atttccgccc ggatccgggc aggttctccg gccgcttggg tggagaggct attcggctat 540
gactgggcac aacagacaat cggctgctct gatgccgccg tgttccggct gtcagcgcag 600
gggcgcccgg ttctttttgt caagaccgac ctgtccggtg ccctgaatga actgcaggac 660
gaggcagcgc ggctatcgtg gctggccacg acgggcgttc cttgcgcagc tgtgctcgac 720
gttgtcactg aagcgggaag ggactggctg ctattgggcg aagtgccggg gcaggatctc 780
ctgtcatctc accttgctcc tgccgagaaa gtatccatca tggctgatgc aatgcggcgg 840
ctgcatacgc ttgatccggc tacctgccca ttcgaccacc aagcgaaaca tcgcatcgag 900
cgagcacgta ctcggatgga agccggtctt gtcgatcagg atgatctgga cgaagagcat 960
caggggctcg cgccagccga actgttcgcc aggctcaagg cgcgcatgcc cgacggcgag 1020
gatctcgtcg tgacccatgg cgatgcctgc ttgccgaata tcatggtgga aaatggccgc 1080
ttttctggat tcatcgactg tggccggctg ggtgtggcgg accgctatca ggacatagcg 1140
ttggctaccc gtgatattgc tgaagagctt ggcggcgaat gggctgaccg cttcctcgtg 1200
ctttacggta tcgccgctcc cgattcgcag cgcatcgcct tctatcgcct tcttgacgag 1260
ttcttctgag cgggactctg gggttcgaaa tgaccgacca agcgacgccc aacctgccat 1320
cacgagattt cgattccacc gccgccttct atgaaaggtt gggcttcgga atcgttttcc 1380
gggacgccgg ctggatgatc ctccagcgcg gggatctcat gctggagttc ttcgcccacc 1440
ccgatccaac acttacgttt gcaacgtcca agagcaaata gaccacgaac gccggaaggt 1500
tgccgcagcg tgtggattgc gtctcaattc tctcttgcag gaatgcaatg atgaatatga 1560
tactgactat gaaactttga gggaatactg cctagcaccg tcacctcata acgtgcatca 1620
tgcatgccct gacaacatgg aacatcgcta tttttctgaa gaattatgct cgttggagga 1680
tgtcgcggca attgcagcta ttgccaacat cgaactaccc ctcacgcatg cattcatcaa 1740
tattattcat gcggggaaag gcaagattaa tccaactggc aaatcatcca gcgtgattgg 1800
taacttcagt tccagcgact tgattcgttt tggtgctacc cacgttttca ataaggacga 1860

gatggtggag taaagaagga gtgcgtcgaa gcagatcgtt caaacatttg gcaataaagt 1920
ttcttaagat tgaatcctgt tgccggtctt gcgatgatta tcatataatt tctgttgaat 1980
tacgttaagc atgtaataat taacatgtaa tgcatgacgt tatttatgag atgggttttt 2040
atgattagag tcccgcaatt atacatttaa tacgcgatag aaaacaaaat atagcgcgca 2100
aactaggata aattatcgcg cgcggtgtca tctatgttac tagatcgaat taattccagg 2160
cggtgaaggg caatcagctg ttgcccgtct cactggtgaa aagaaaaacc accccagtac 2220
attaaaaacg tccgcaatgt gttattaagt tgtctaagcg tcaatttgtt tacaccacaa 2280
tatatcctgc caccagccag ccaacagctc cccgaccggc agctcggcac aaaatcacca 2340
ctcgatacag gcagcccatc agtccgggac ggcgtcagcg ggagagccgt tgtaaggcgg 2400
cagactttgc tcatgttacc gatgctattc ggaagaacgg caactaagct gccgggtttg 2460
aaacacggat gatctcgcgg agggtagcat gttgattgta acgatgacag agcgttgctg 2520
cctgtgatca aatatcatct ccctcgcaga gatccgaatt atcagccttc ttattcattt 2580
ctcgcttaac cgtgacaggc tgtcgatctt gagaactatg ccgacataat aggaaatcgc 2640
tggataaagc cgctgaggaa gctgagtggc gctatttctt tagaagtgaa cgttgacgat 2700
gtcgacggat cttttccgct gcataaccct gcttcggggt cattatagcg atttttttcgg 2760
tatatccatc ctttttcgca cgatatacag gattttgcca aagggttcgt gtagactttc 2820
cttggtgtat ccaacggcgt cagccgggca ggataggtga agtaggccca cccgcgagcg 2880
ggtgttcctt cttcactgtc ccttattcgc acctggcggt gctcaacggg aatcctgctc 2940
tgcgaggctg gccggctacc gccggcgtaa cagatgaggg caagcggatg gctgatgaaa 3000
ccaagccaac caggggtgat gctgccaact tactgattta gtgtatgatg gtgtttttga 3060
ggtgctccag tggcttctgt ttctatcagc tgtccctcct gttcagctac tgacggggtg 3120
gtgcgtaacg gcaaaagcac cgccggacat cagcgctatc tctgctctca ctgccgtaaa 3180
acatggcaac tgcagttcac ttacaccgct tctcaacccg gtacgcacca gaaaatcatt 3240
gatatggcca tgaatggcgt tggatgccgg gcaacagccc gcattatggg cgttggcctc 3300
aacacgattt tacgtcactt aaaaaactca ggccgcagtc ggtaacctcg cgcatacagc 3360
cgggcagtga cgtcatcgtc tgcgcggaaa tggacgaaca gtggggctat gtcggggcta 3420
aatcgcgcca gcgctggctg ttttacgcgt atgacagtct ccggaagacg gttgttgcgc 3480
acgtattcgg tgaacgcact atggcgacgc tggggcgtct tatgagcctg ctgtcaccct 3540
ttgacgtggt gatatggatg acggatggct ggccgctgta tgaatcccgc ctgaagggaa 3600
agctgcacgt aatcagcaag cgatatacgc agcgaattga gcggcataac ctgaatctga 3660
ggcagcacct ggcacggctg ggacggaagt cgctgtcgtt ctcaaaatcg gtggagctgc 3720

atgacaaagt catcgggcat tatctgaaca taaaacacta tcaataagtt ggagtcatta 3780
cccaaccagg aagggcagcc cacctatcaa ggtgtactgc cttccagacg aacgaagagc 3840
gattgaggaa aaggcggcgg cggccggcat gagcctgtcg gcctacctgc tggccgtcgg 3900
ccagggctac aaaatcacgg gcgtcgtgga ctatgagcac gtccgcgagc tggcccgcat 3960
caatggcgac ctgggccgcc tgggcggcct gctgaaactc tggctcaccg acgacccgcg 4020
cacggcgcgg ttcggtgatg ccacgatcct cgccctgctg gcgaagatcg aagagaagca 4080
ggacgagctt ggcaaggtca tgatgggcgt ggtccgcccg agggcagagc catgactttt 4140
ttagccgcta aaacggccgg ggggtgcgcg tgattgccaa gcacgtcccc atgcgctcca 4200
tcaagaagag cgacttcgcg gagctggtat tcgtgcaggg caagattcgg aataccaagt 4260
acgagaagga cggccagacg gtctacggga ccgacttcat tgccgataag gtggattatc 4320
tggacaccaa ggcaccaggc gggtcaaatc aggaataagg gcacattgcc ccggcgtgag 4380
tcggggcaat cccgcaagga gggtgaatga atcggacgtt tgaccggaag gcatacaggc 4440
aagaactgat cgacgcgggg ttttccgccg aggatgccga aaccatcgca agccgcaccg 4500
tcatgcgtgc gccccgcgaa accttccagt ccgtcggctc gatggtccag caagctacgg 4560
ccaagatcga gcgcgacagc gtgcaactgg ctcccctgc cctgcccgcg ccatcggccg 4620
ccgtggagcg ttcgcgtcgt ctcgaacagg aggcggcagg tttggcgaag tcgatgacca 4680
tcgacacgcg aggaactatg acgaccaaga agcgaaaaac cgccggcgag gacctggcaa 4740
aacaggtcag cgaggccaag caggccgcgt tgctgaaaca cacgaagcag cagatcaagg 4800
aaatgcagct ttccttgttc gatattgcgc cgtggccgga cacgatgcga gcgatgccaa 4860
acgacacggc ccgctctgcc ctgttcacca cgcgcaacaa gaaaatcccg cgcgaggcgc 4920
tgcaaaacaa ggtcattttc cacgtcaaca aggacgtgaa gatcacctac accggcgtcg 4980
agctgcgggc cgacgatgac gaactggtgt ggcagcaggt gttggagtac gcgaagcgca 5040
cccctatcgg cgagccgatc accttcacgt tctacgagct ttgccaggac ctgggctggt 5100
cgatcaatgg ccggtattac acgaaggccg aggaatgcct gtcgcgccta caggcgacgg 5160
cgatgggctt cacgtccgac cgcgttgggc acctggaatc ggtgtcgctg ctgcaccgct 5220
tccgcgtcct ggaccgtggc aagaaaacgt cccgttgcca ggtcctgatc gacgaggaaa 5280
tcgtcgtgct gtttgctggc gaccactaca cgaaattcat atgggagaag taccgcaagc 5340
tgtcgccgac ggcccgacgg atgttcgact atttcagctc gcaccgggag ccgtacccgc 5400
tcaagctgga aaccttccgc ctcatgtgcg gatcggattc cacccgcgtg aagaagtggc 5460
gcgagcaggt cggcgaagcc tgcgaagagt tgcgaggcag cggcctggtg gaacacgcct 5520
gggtcaatga tgacctggtg cattgcaaac gctagggcct tgtggggtca gttccggctg 5580

ggggttcagc agccagcgct ttactggcat ttcaggaaca agcgggcact gctcgacgca 5640
cttgcttcgc tcagtatcgc tcgggacgca cggcgcgctc tacgaactgc cgataaacag 5700
aggattaaaa ttgacaattg tgattaaggc tcagattcga cggcttggag cggccgacgt 5760
gcaggatttc cgcgagatcc gattgtcggc cctgaagaaa gctccagaga tgttcgggtc 5820
cgtttacgag cacgaggaga aaaagcccat ggaggcgttc gctgaacggt tgcgagatgc 5880
cgtggcattc ggcgcctaca tcgacggcga gatcattggg ctgtcggtct tcaaacagga 5940
ggacggcccc aaggacgctc acaaggcgca tctgtccggc gttttcgtgg agcccgaaca 6000
gcgaggccga ggggtcgccg gtatgctgct gcgggcgttg ccggcgggtt tattgctcgt 6060
gatgatcgtc cgacagattc caacgggaat ctggtggatg cgcatcttca tcctcggcgc 6120
acttaatatt tcgctattct ggagcttgtt gtttatttcg gtctaccgcc tgccgggcgg 6180
ggtcgcggcg acggtaggcg ctgtgcagcc gctgatggtc gtgttcatct ctgccgctct 6240
gctaggtagc ccgatacgat tgatggcggt cctgggggct atttgcggaa ctgcgggcgt 6300
ggcgctgttg gtgttgacac caaacgcagc gctagatcct gtcggcgtcg cagcgggcct 6360
ggcgggggcg gtttccatgg cgttcggaac cgtgctgacc cgcaagtggc aacctcccgt 6420
gcctctgctc acctttaccg cctggcaact ggcggccgga ggacttctgc tcgttccagt 6480
agctttagtg tttgatccgc caatcccgat gcctacagga accaatgttc tcggcctggc 6540
gtggctcggc ctgatcggag cgggtttaac ctacttcctt tggttccggg ggatctcgcg 6600
actcgaacct acagttgttt ccttactggg ctttctcagc cgggatggcg ctaagaagct 6660
attgccgccg atcttcatat gcggtgtgaa ataccgcaca gatgcgtaag gagaaaatac 6720
cgcatcaggc gctcttccgc ttcctcgctc actgactcgc tgcgctcggt cgttcggctg 6780
cggcgagcgg tatcagctca ctcaaaggcg gtaatacggt tatccacaga atcaggggat 6840
aacgcaggaa agaacatgtg agcaaaaggc cagcaaaagg ccaggaaccg taaaaaggcc 6900
gcgttgctgg cgtttttcca taggctccgc ccccctgacg agcatcacaa aaatcgacgc 6960
tcaagtcaga ggtggcgaaa cccgacagga ctataaagat accaggcgtt tccccctgga 7020
agctccctcg tgcgctctcc tgttccgacc ctgccgctta ccggatacct gtccgccttt 7080
ctcccttcgg gaagcgtggc gctttctcaa tgctcacgct gtaggtatct cagttcggtg 7140
taggtcgttc gctccaagct gggctgtgtg cacgaacccc ccgttcagcc cgaccgctgc 7200
gccttatccg gtaactatcg tcttgagtcc aacccggtaa gacacgactt atcgccactg 7260
gcagcagcca ctggtaacag gattagcaga gcgaggtatg taggcggtgc tacagagttc 7320
ttgaagtggt ggcctaacta cggctacact agaaggacag tatttggtat ctgcgctctg 7380
ctgaagccag ttaccttcgg aaaaagagtt ggtagctctt gatccggcaa acaaaccacc 7440

gctggtagcg gtggtttttt tgtttgcaag cagcagatta cgcgcagaaa aaaaggatat 7500
caagaagatc ctttgatctt ttctacgggg tctgacgctc agtggaacga aaactcacgt 7560
taagggattt tggtcatgag attatcaaaa aggatcttca cctagatcct tttaaattaa 7620
aaatgaagtt ttaaatcaat ctaaagtata tatgagtaaa cttggtctga cagttaccaa 7680
tgcttaatca gtgaggcacc tatctcagcg atctgtctat ttcgttcatc catagttgcc 7740
tgactccccg tcgtgtagat aactacgata cgggagggct taccatctgg ccccagtgct 7800
gcaatgatac cgcgagaccc acgctcaccg gctccagatt tatcagcaat aaaccagcca 7860
gccggaaggg ccgagcgcag aagtggtcct gcaactttat ccgcctccat ccagtctatt 7920
aaacaagtgg cagcaacgga ttcgcaaacc tgtcacgcct tttgtgccaa aagccgcgcc 7980
aggtttgcga tccgctgtgc caggcgttag gcgtcatatg aagatttcgg tgatccctga 8040
gcaggtggcg gaaacattgg atgctgagaa ccatttcatt gttcgtgaag tgttcgatgt 8100
gcacctatcc gaccaaggct ttgaactatc taccagaagt gtgagcccct accggaagga 8160
ttacatctcg gatgatgact ctgatgaaga ctctgcttgc tatggcgcat tcatcgacca 8220
agagcttgtc gggaagattg aactcaactc aacatggaac gatctagcct ctatcgaaca 8280
cattgttgtg tcgcacacgc accgaggcaa aggagtcgcg cacagtctca tcgaatttgc 8340
gaaaaagtgg gcactaagca gacagctcct tggcatacga ttagagacac aaacgaacaa 8400
tgtacctgcc tgcaatttgt acgcaaaatg tggctttact ctcggcggca ttgacctgtt 8460
cacgtataaa actagacctc aagtctcgaa cgaaacagcg atgtactggt actggttctc 8520
gggagcacag gatgacgcct aacaattcat tcaagccgac accgcttcgc ggcgcggctt 8580
aattcaggag ttaaacatca tgagggaagc ggtgatcgcc gaagtatcga ctcaactatc 8640
agaggtagtt ggcgtcatcg agcgccatct cgaaccgacg ttgctggccg tacatttgta 8700
cggctccgca gtggatggcg gcctgaagcc acacagtgat attgatttgc tggttacggt 8760
gaccgtaagg cttgatgaaa caacgcggcg agctttgatc aacgaccttt tggaaacttc 8820
ggcttcccct ggagagagcg agattctccg cgctgtagaa gtcaccattg ttgtgcacga 8880
cgacatcatt ccgtggcgtt atccagctaa gcgcgaactg caatttggag aatggcagcg 8940
caatgacatt cttgcaggta tcttcgagcc agccacgatc gacattgatc tggctatctt 9000
gctgacaaaa gcaagagaac atagcgttgc cttggtaggt ccagcggcgg aggaactctt 9060
tgatccggtt cctgaacagg atctatttga ggcgctaaat gaaaccttaa cgctatggaa 9120
ctcgccgccc gactgggctg gcgatgagcg aaatgtagtg cttacgttgt cccgcatttg 9180
gtacagcgca gtaaccggca aaatcgcgcc gaaggatgtc gctgccgact gggcaatgga 9240
gcgcctgccg gcccagtatc agcccgtcat acttgaagct aggcaggctt atcttggaca 9300

agaagatcgc ttggcctcgc gcgcagatca gttggaagaa tttgttcact acgtgaaagg 9360
cgagatcacc aaggtagtcg gcaaataatg tctaacaatt cgttcaagcc gacgccgctt 9420
cgcggcgcgg cttaactcaa gcgttagaga gctggggaag actatgcgcg atctgttgaa 9480
ggtggttcta agcctcgtac ttgcgatggc atcggggcag gcacttgctg acctgccaat 9540
tgttttagtg gatgaagctc gtcttcccta tgactactcc ccatccaact acgacatttc 9600
tccaagcaac tacgacaact ccataagcaa ttacgacaat agtccatcaa attacgacaa 9660
ctctgagagc aactacgata atagttcatc caattacgac aatagtcgca acggaaatcg 9720
taggcttata tatagcgcaa atgggtctcg cactttcgcc ggctactacg tcattgccaa 9780
caatgggaca acgaacttct tttccacatc tggcaaaagg atgttctaca ccccaaaagg 9840
ggggcgcggc gtctatggcg gcaaagatgg gagcttctgc ggggcattgg tcgtcataaa 9900
tggccaattt tcgcttgccc tgacagataa cggcctgaag atcatgtatc taagcaacta 9960
gcctgctctc taataaaatg ttaggagctt ggctgccatt tttggggtga ggccgttcgc 10020
ggccgagggg cgcagcccct gggggggatgg gaggcccgcg ttagcgggcc gggagggttc 10080
gagaaggggg ggcacccccc ttcggcgtgc gcggtcacgc gccagggcgc agccctggtt 10140
aaaaacaagg tttataaata ttggtttaaa agcaggttaa aagacaggtt agcggtggcc 10200
gaaaaacggg cggaaaccct tgcaaatgct ggattttctg cctgtggaca gcccctcaaa 10260
tgtcaatagg tgcgcccctc atctgtcagc actctgcccc tcaagtgtca aggatcgcgc 10320
ccctcatctg tcagtagtcg cgcccctcaa gtgtcaatac cgcagggcac ttatccccag 10380
gcttgtccac atcatctgtg ggaaactcgc gtaaaatcag gcgttttcgc cgatttgcga 10440
ggctggccag ctccacgtcg ccggccgaaa tcgagcctgc ccctcatctg tcaacgccgc 10500
gccgggtgag tcggcccctc aagtgtcaac gtccgcccct catctgtcag tgagggccaa 10560
gttttccgcg aggtatccac aacgccggcg gccggccgcg gtgtctcgca cacggcttcg 10620
acggcgtttc tggcgcgttt gcagggccat agacggccgc cagcccagcg gcgagggcaa 10680
ccagcccggt gagcgtcgga aagggtcgac atcttgctgc gttcggatat tttcgtggag 10740
ttcccgccac agacccggat tgaaggcgag atccagcaac tcgcgccaga tcatcctgtg 10800
acggaacttt ggcgcgtgat gactggccag gacgtcggcc gaaagagcga caagcagatc 10860
acgattttcg acagcgtcgg atttgcgatc gaggattttt cggcgctgcg ctacgtccgc 10920
gaccgcgttg agggatcaag ccacagcagc ccactcgacc ttctagccga cccagacgag 10980
ccaagggatc tttttggaat gctgctccgt cgtcaggctt tccgacgttt gggtggttga 11040
acagaagtca ttatcgtacg gaatgccagc actcccgagg ggaaccctgt ggttggcatg 11100
cacatacaaa tggacgaacg gataaacctt ttcacgccct tttaaatatc cgttattcta 11160

ataaacgctc ttttctctta ggtttacccg ccaatatatc ctgtcaaaca ctgatagttt 11220
aaactgaagg cgggaaacga caatctgatc atgagcggag aattaaggga gtcacgttat 11280
gaccccccgcc gatgacgcgg gacaagccgt tttacgtttg gaactgacag aaccgcaacg 11340
attgaaggag ccactcagcc ccaatacgca aaccgcctct ccccgcgcgt tggccgattc 11400
attaatgcag ctggcacgac aggtttcccg actggaaagc gggcagtgag cgcaacgcaa 11460
ttaatgtgag ttagctcact cattaggcac cccaggcttt acactttatg cttccggctc 11520
gtatgttgtg tggaattgtg agcggataac aatttcacac aggaaacagc tatgaccatg 11580
attacgccaa gctatttagg tgacactata gaatactcaa gctatgcatc caacgcgttg 11640
ggagctctcc catatcgacc tgcaggcggc cgctcgacga attaattcca atcccacaaa 11700
aatctgagct taacagcaca gttgctcctc tcagagcaga atcgggtatt caacaccctc 11760
atatcaacta ctacgttgtg tataacggtc cacatgccgg tatatacgat gactggggtt 11820
gtacaaaggc ggcaacaaac ggcgttcccg gagttgcaca caagaaattt gccactatta 11880
cagaggcaag agcagcagct gacgcgtaca caacaagtca gcaaacagac aggttgaact 11940
tcatccccaa aggagaagct caactcaagc ccaagagctt tgctaaggcc ctaacaagcc 12000
caccaaagca aaaagcccac tggctcacgc taggaaccaa aaggcccagc agtgatccag 12060
ccccaaaaga gatctccttt gccccggaga ttacaatgga cgatttcctc tatctttacg 12120
atctaggaag gaagttcgaa ggtgaaggtg acgacactat gttcaccact gataatgaga 12180
aggttagcct cttcaatttc agaaagaatg ctgacccaca gatggttaga gaggcctacg 12240
cagcaggtct catcaagacg atctacccga gtaacaatct ccaggagatc aaataccttc 12300
ccaagaaggt taaagatgca gtcaaaagat tcaggactaa ttgcatcaag aacacagaga 12360
aagacatatt tctcaagatc agaagtacta ttccagtatg gacgattcaa ggcttgcttc 12420
ataaaccaag gcaagtaata gagattggag tctctaaaaa ggtagttcct actgaatcta 12480
aggccatgca tggagtctaa gattcaaatc gaggatctaa cagaactcgc cgtgaagact 12540
ggcgaacagt tcatacagag tcttttacga ctcaatgaca agaagaaaat cttcgtcaac 12600
atggtggagc acgacactct ggtctactcc aaaaatgtca aagatacagt ctcagaagac 12660
caaagggcta ttgagacttt tcaacaaagg ataatttcgg gaaacctcct cggattccat 12720
tgcccagcta tctgtcactt catcgaaagg acagtagaaa aggaaggtgg ctcctacaaa 12780
tgccatcatt gcgataaagg aaaggctatc attcaagatc tctctgccga cagtggtccc 12840
aaagatggac ccccacccac gaggagcatc gtggaaaaag aagacgttcc aaccacgtct 12900
tcaaagcaag tggattgatg tgacatctcc actgacgtaa gggatgacgc acaatcccac 12960
tatccttcgc aagacccttc ctctatataa ggaagttcat ttcatttgga gaggacacgc 13020

tcgaggctag catggatctc gggccccaaa taatgatttt attttgactg atagtgacct 13080
gttcgttgca acaaattgat gagcaatgct tttttataat gccaactttg tacaaaaaag 13140
ctgaacgaga aacgtaaaat gatataaata tcaatatatt aaattagatt ttgcataaaa 13200
aacagactac ataatactgt aaaacacaac atatccagtc actatgaatc aactacttag 13260
atggtattag tgacctgtag tcgaccgaca gccttccaaa tgttcttcgg gtgatgctgc 13320
caacttagtc gaccgacagc cttccaaatg ttcttctcaa acggaatcgt cgtatccagc 13380
ctactcgcta ttgtcctcaa tgccgtatta aatcataaaa agaaataaga aaaagaggtg 13440
cgagcctctt ttttgtgtga caaaataaaa acatctacct attcatatac gctagtgtca 13500
tagtcctgaa aatcatctgc atcaagaaca atttcacaac tcttatactt ttctcttaca 13560
agtcgttcgg cttcatctgg attttcagcc tctatactta ctaaacgtga taaagtttct 13620
gtaatttcta ctgtatcgac ctgcagactg gctgtgtata agggagcctg acatttatat 13680
tccccagaac atcaggttaa tggcgttttt gatgtcattt tcgcggtggc tgagatcagc 13740
cacttcttcc ccgataacgg agaccggcac actggccata tcggtggtca tcatgcgcca 13800
gctttcatcc ccgatatgca ccaccgggta aagttcacgg gagactttat ctgacagcag 13860
acgtgcactg gccaggggga tcaccatccg tcgcccgggc gtgtcaataa tatcactctg 13920
tacatccaca aacagacgat aacggctctc tcttttatag gtgtaaacct taaactgcat 13980
ttcaccagtc cctgttctcg tcagcaaaag agccgttcat ttcaataaac cgggcgacct 14040
cagccatccc ttcctgattt tccgctttcc agcgttcggc acgcagacga cgggcttcat 14100
tctgcatggt tgtgcttacc agaccggaga tattgacatc atatatgcct tgagcaactg 14160
atagctgtcg ctgtcaactg tcactgtaat acgctgcttc atagcacacc tctttttgac 14220
atacttcggg tagtgccgat caacgtctca ttttcgccaa aagttggccc agggcttccc 14280
ggtatcaaca gggacaccag gatttattta ttctgcgaag tgatcttccg tcacaggtat 14340
ttattcggcg caaagtgcgt cgggtgatgc tgccaactta gtcgactaca ggtcactaat 14400
accatctaag tagttgattc atagtgactg gatatgttgt gttttacagt attatgtagt 14460
ctgtttttta tgcaaaatct aatttaatat attgatattt atatcatttt acgtttctcg 14520
ttcagctttc ttgtacaaag ttggcattat aagaaagcat tgcttatcaa tttgttgcaa 14580
cgaacaggtc actatcagtc aaaataaaat cattatttgc catccagctg cagctcctcg 14640
aggaattcgg taccccaatt ggtaaggaaa taattatttt ctttttttcct tttagtataa 14700
aatagttaag tgatgttaat tagtatgatt ataataatat agttgttata attgtgaaaa 14760
aataatttat aaatatattg tttacataaa caacatagta atgtaaaaaa atatgacaag 14820
tgatgtgtaa gacgaagaag ataaaagttg agagtaagta tattattttt aatgaatttg 14880

atcgaacatg taagatgata tacggccggt aagaggttcc aactttcacc ataatgaaat 14940
aagatcacta ccgggcgtat tttttgagtt atcgagattt tcaggagcta aggaagctaa 15000
aatggagaaa aaaatcactg gatataccac cgttgatata tcccaatggc atcgtaaaga 15060
acattttgag gcatttcagt cagttgctca atgtacctat aaccagaccg ttcagctgga 15120
tattacggcc tttttaaaga ccgtaaagaa aaataagcac aagttttatc cggcctttat 15180
tcacattctt gcccgcctga tgaatgctca tccggaattc cgtatggcaa tgaaagacgg 15240
tgagctggtg atatgggata gtgttcaccc ttgttacacc gttttccatg agcaaactga 15300
aacgttttca tcgctctgga gtgaatacca cgacgatttc cggcagtttc tacacatata 15360
ttcgcaagat gtggcgtgtt acggtgaaaa cctggcctat ttccctaaag ggtttattga 15420
gaatatgttt ttcgtctcag ccaatccctg ggtgagtttc accagttttg atttaaacgt 15480
ggccaatatg gacaacttct tcgcccccgt tttcaccatg ggcaaatatt atacgcaagg 15540
cgacaaggtg ctgatgccgc tggcgattca ggttcatcat gccgtctgtg atggcttcca 15600
tgtcggcaga atgcttaatg aattacaaca gtactgcgat gagtggcagg gcggggcgta 15660
atcgcgtgga tccggcttac taaaagccag ataacagtat gcgtatttgc gcgctgattt 15720
ttgcggtata agaatatata ctgatatgtc gggcccataa tagtaattct agctggtttg 15780
atgaattaaa tatcaatgat aaaatactat agtaaaaata agaataaata aattaaaata 15840
atattttttt atgattaata gtttattata taattaaata tctataccat tactaaatat 15900
tttagtttaa aagttaataa atattttgtt agaaattcca atctgcttgt aatttatcaa 15960
taaacaaaat attaaataac aagctaaagt aacaaataat atcaaactaa tagaaacagt 16020
aatctaatgt aacaaaacat aatctaatgc taatataaca aagcgcaaga tctatcattt 16080
tatatagtat tattttcaat caacattctt attaatttct aaataatact tgtagtttta 16140
ttaacttcta aatggattga ctattaatta aatgaattag tcgaacatga ataaacaagg 16200
taacatgata gatcatgtca ttgtgttatc attgatctta catttggatt gattacagtt 16260
gggaaattgg gttcgaaatc gataagcttg gatcctctag agagctgcag ctggatggca 16320
aataatgatt ttattttgac tgatagtgac ctgttcgttg caacaaattg ataagcaatg 16380
ctttcttata atgccaactt tgtacaagaa agctgaacga gaaacgtaaa atgatataaa 16440
tatcaatata ttaaattaga ttttgcataa aaaacagact acataatact gtaaaacaca 16500
acatatccag tcactatgaa tcaactactt agatggtatt agtgacctgt agtcgactaa 16560
gttggcagca tcacccgacg cactttgcgc cgaataaata cctgtgacgg aagatcactt 16620
cgcagaataa ataaatcctg gtgtccctgt tgataccggg aagccctggg ccaacttttg 16680
gcgaaaatga gacgttgatc ggcactaccc atttcacaac tcttatactt ttctcttaca 16740

agtcgttcgg cttcatctgg attttcagcc tctatactta ctaaacgtga taaagtttct 16800
gtaatttcta ctgtatcgac ctgcagactg gctgtgtata agggagcctg acatttatat 16860
tccccagaac atcaggttaa tggcgttttt gatgtcattt tcgcggtggc tgagatcagc 16920
cacttcttcc ccgataacgg agaccggcac actggccata tcggtggtca tcatgcgcca 16980
gctttcatcc ccgatatgca ccaccgggta aagttcacgg gagactttat ctgacagcag 17040
acgtgcactg gccaggggga tcaccatccg tcgcccgggc gtgtcaataa tatcactctg 17100
tacatccaca aacagacgat aacggctctc tcttttatag gtgtaaacct taaactgcat 17160
ttcaccagtc cctgttctcg tcagcaaaag agccgttcat ttcaataaac cgggcgacct 17220
cagccatccc ttcctgattt tccgctttcc agcgttcggc acgcagacga cgggcttcat 17280
tctgcatggt tgtgcttacc agaccggaga tattgacatc atatatgcct tgagcaactg 17340
atagctgtcg ctgtcaactg tcactgtaat acgctgcttc atagcacacc tctttttgac 17400
atacttctgt tcttgatgca gatgattttc aggactatga cactagcgta tatgaatagg 17460
tagatgtttt tattttgtca cacaaaaaag aggctcgcac ctcttttttct tatttctttt 17520
tatgatttaa tacggcattg aggacaatag cgagtaggct ggatacgacg attccgtttg 17580
agaagaacat ttggaaggct gtcggtcgac taagttggca gcatcacccg aagaacattt 17640
ggaaggctgt cggtcgacta caggtcacta ataccatcta agtagttgat tcatagtgac 17700
tggatatgtt gtgttttaca gtattatgta gtctgttttt tatgcaaaat ctaatttaat 17760
atattgatat ttatatcatt ttacgtttct cgttcagctt ttttgtacaa agttggcatt 17820
ataaaaaagc attgctcatc aatttgttgc aacgaacagg tcactatcag tcaaaataaa 17880
atcattattt ggggcccgag atccatgcta gctctagagt cctgctttaa tgagatatgc 17940
gagacgccta tgatcgcatg atatttgctt tcaattctgt tgtgcacgtt gtaaaaaacc 18000
tgagcatgtg tagctcagat ccttaccgcc ggtttcggtt cattctaatg aatatatcac 18060
ccgttactat cgtattttta tgaataatat tctccgttca atttactgat tgtaccctac 18120
tacttatatg tacaatatta aaatgaaaac aatatattgt gctgaatagg tttatagcga 18180
catctatgat agagcgccac aataacaaac aattgcgttt tattattaca aatccaattt 18240
taaaaaaagc ggcagaaccg gtcaaaccta aaagactgat tacataaatc ttattcaaat 18300
ttcaaaaggc cccaggggct agtatctacg acacaccgag cggcgaacta ataacgttca 18360
ctgaagggaa ctccggttcc ccgccggcgc gcatgggtga gattccttga agttgagtat 18420
tggccgtccg ctctaccgaa agttacgggc accattcaac ccggtccagc acggcggccg 18480
ggtaaccgac ttgctgcccc gagaattatg cagcattttt ttggtgtatg tgggccccaa 18540
atgaagtgca ggtcaaacct tgacagtgac gacaaatcgt tgggcgggtc cagggcgaat 18600

tttgcgacaa catgtcgagg ctcagcagga cctgcaggca tgcaagctag cttactagtg 18660

atgcatattc tatagtgtca cctaaatctg c 18691

<210> 14
<211> 59
<212> DNA
<213> Artificial sequence

<220>
<223> forward primer used for the amplification of 200 and 400 bp CHS f
ragments

<400> 14
ggggacaagt ttgtacaaaa aagcaggctg cactgctaac cctgagaacc atgtgcttc 59

<210> 15
<211> 59
<212> DNA
<213> Artificial sequence

<220>
<223> reverse primer for amplification of 400 bp CHS fragment

<400> 15
ggggaccact ttgtacaaga aagctgggtc gcttgacgga aggacggaga ccaagaagc 59

<210> 16
<211> 59
<212> DNA
<213> Artificial sequence

<220>
<223> reverse primer for amplification of 200bp CHS fragment

<400> 16
ggggaccact ttgtacaaga aagctgggta ggagccatgt aagcacacat gtgtgggtt 59

<210> 17
<211> 100
<212> DNA
<213> Artificial sequence

<220>
<223> forward primer for amplification of 100bp CHS fragment

<400> 17
ggggacaagt ttgtacaaaa aagcaggctg cactgctaac cctgagaacc atgtgcttca 60

ggcggagtat cctgactact acttccgcat caccaacagt 100

<210> 18
<211> 100
<212> DNA
<213> Artificial sequence

<220>

<223> reverse primer for amplification of 100 bp CHS fragment

<400> 18
ggggaccact ttgtacaaga aagctgggta acttctcctt gaggtcggtc atgtgttcac 60

tgttggtgat gcggaagtag tagtcaggat actccgcctg 100

<210> 19
<211> 79
<212> DNA
<213> Artificial sequence

<220>
<223> forward primer for amplification of 50 bp CHS fragment

<400> 19
ggggacaagt ttgtacaaaa aagcaggctg cactgctaac cctgagaacc atgtgcttca 60

ggcggagtat cctgactac 79

<210> 20
<211> 79
<212> DNA
<213> Artificial sequence

<220>
<223> reverse primer for 50 bp CHS fragment

<400> 20
ggggaccact ttgtacaaga aagctgggtg tagtcaggat actccgcctg aagcacatgg 60

ttctcagggt tagcagtgc 79

<210> 21
<211> 54
<212> DNA
<213> Artificial sequence

<220>
<223> forward primer for amplification of the 25 bp CHS fragment

<400> 21
ggggacaagt ttgtacaaaa aagcaggctg cactgctaac cctgagaacc atgt 54

<210> 22
<211> 54
<212> DNA
<213> Artificial sequence

<220>
<223> reverse primer for amplification of the 25 bp CHS fragment

<400> 22
ggggaccact ttgtacaaga aagctgggta catggttctc agggttagca gtgc 54

<210> 23
<211> 15

<212> DNA
<213> Artificial sequence

<220>
<223> acceptor vector pHELLSGATE4

<400> 23
aaaaaaaaaa aaaaa 15

<210> 24
<211> 17476
<212> DNA
<213> Artificial sequence

<220>
<223> acceptor vector pHELLSGATE8

<400> 24
ggccgcacta gtgatatccc gcggccatgg cggccgggag catgcgacgt cgggcccaat 60
tcgccctata gtgagtcgta ttacaattca ctggccgtcg ttttacaacg tcgtgactgg 120
gaaaaccctg gcgttaccca acttaatcgc cttgcagcac atcccccttt cgccagctgg 180
cgtaatagcg aagaggcccg caccgatcgc ccttcccaac agttgcgcag cctgaatggc 240
gaatggaaat tgtaaacgtt aatgggtttc tggagtttaa tgagctaagc acatacgtca 300
gaaaccatta ttgcgcgttc aaaagtcgcc taaggtcact atcagctagc aaatatttct 360
tgtcaaaaat gctccactga cgttccataa attcccctcg gtatccaatt agagtctcat 420
attcactctc aatccaaata atctgcaatg gcaattacct tatccgcaac ttctttacct 480
atttccgccc ggatccgggc aggttctccg gccgcttggg tggagaggct attcggctat 540
gactgggcac aacagacaat cggctgctct gatgccgccg tgttccggct gtcagcgcag 600
gggcgcccgg ttctttttgt caagaccgac ctgtccggtg ccctgaatga actgcaggac 660
gaggcagcgc ggctatcgtg gctggccacg acgggcgttc cttgcgcagc tgtgctcgac 720
gttgtcactg aagcgggaag ggactggctg ctattgggcg aagtgccggg gcaggatctc 780
ctgtcatctc accttgctcc tgccgagaaa gtatccatca tggctgatgc aatgcggcgg 840
ctgcatacgc ttgatccggc tacctgccca ttcgaccacc aagcgaaaca tcgcatcgag 900
cgagcacgta ctcggatgga agccggtctt gtcgatcagg atgatctgga cgaagagcat 960
caggggctcg cgccagccga actgttcgcc aggctcaagg cgcgcatgcc cgacggcgag 1020
gatctcgtcg tgacccatgg cgatgcctgc ttgccgaata tcatggtgga aaatggccgc 1080
ttttctggat tcatcgactg tggccggctg ggtgtggcgg accgctatca ggacatagcg 1140
ttggctaccc gtgatattgc tgaagagctt ggcggcgaat gggctgaccg cttcctcgtg 1200
ctttacggta tcgccgctcc cgattcgcag cgcatcgcct tctatcgcct tcttgacgag 1260
ttcttctgag cgggactctg gggttcgaaa tgaccgacca agcgacgccc aacctgccat 1320

cacgagattt cgattccacc gccgccttct atgaaaggtt gggcttcgga atcgttttcc 1380
gggacgccgg ctggatgatc ctccagcgcg gggatctcat gctggagttc ttcgcccacc 1440
ccgatccaac acttacgttt gcaacgtcca agagcaaata gaccacgaac gccggaaggt 1500
tgccgcagcg tgtggattgc gtctcaattc tctcttgcag gaatgcaatg atgaatatga 1560
tactgactat gaaactttga gggaatactg cctagcaccg tcacctcata acgtgcatca 1620
tgcatgccct gacaacatgg aacatcgcta tttttctgaa gaattatgct cgttggagga 1680
tgtcgcggca attgcagcta ttgccaacat cgaactaccc ctcacgcatg cattcatcaa 1740
tattattcat gcgggggaaag gcaagattaa tccaactggc aaatcatcca gcgtgattgg 1800
taacttcagt tccagcgact tgattcgttt tggtgctacc cacgttttca ataaggacga 1860
gatggtggag taaagaagga gtgcgtcgaa gcagatcgtt caaacatttg gcaataaagt 1920
ttcttaagat tgaatcctgt tgccggtctt gcgatgatta tcatataatt tctgttgaat 1980
tacgttaagc atgtaataat taacatgtaa tgcatgacgt tatttatgag atgggttttt 2040
atgattagag tcccgcaatt atacatttaa tacgcgatag aaaacaaaat atagcgcgca 2100
aactaggata aattatcgcg cgcggtgtca tctatgttac tagatcgaat taattccagg 2160
cggtgaaggg caatcagctg ttgcccgtct cactggtgaa aagaaaaacc accccagtac 2220
attaaaaacg tccgcaatgt gttattaagt tgtctaagcg tcaatttgtt tacaccacaa 2280
tatatcctgc caccagccag ccaacagctc cccgaccggc agctcggcac aaaatcacca 2340
ctcgatacag gcagcccatc agtccgggac ggcgtcagcg ggagagccgt tgtaaggcgg 2400
cagactttgc tcatgttacc gatgctattc ggaagaacgg caactaagct gccgggtttg 2460
aaacacggat gatctcgcgg agggtagcat gttgattgta acgatgacag agcgttgctg 2520
cctgtgatca aatatcatct ccctcgcaga gatccgaatt atcagccttc ttattcattt 2580
ctcgcttaac cgtgacaggc tgtcgatctt gagaactatg ccgacataat aggaaatcgc 2640
tggataaagc cgctgaggaa gctgagtggc gctatttctt tagaagtgaa cgttgacgat 2700
gtcgacggat cttttccgct gcataaccct gcttcggggt cattatagcg atttttcgg 2760
tatatccatc ctttttcgca cgatatacag gattttgcca aagggttcgt gtagactttc 2820
cttggtgtat ccaacggcgt cagccgggca ggataggtga agtaggccca cccgcgagcg 2880
ggtgttcctt cttcactgtc ccttattcgc acctggcggt gctcaacggg aatcctgctc 2940
tgcgaggctg gccggctacc gccggcgtaa cagatgaggg caagcggatg gctgatgaaa 3000
ccaagccaac caggggtgat gctgccaact tactgattta gtgtatgatg gtgtttttga 3060
ggtgctccag tggcttctgt ttctatcagc tgtccctcct gttcagctac tgacggggtg 3120
gtgcgtaacg gcaaaagcac cgccggacat cagcgctatc tctgctctca ctgccgtaaa 3180

acatggcaac tgcagttcac ttacaccgct tctcaacccg gtacgcacca gaaaatcatt 3240

gatatggcca tgaatggcgt tggatgccgg gcaacagccc gcattatggg cgttggcctc 3300

aacacgattt tacgtcactt aaaaaactca ggccgcagtc ggtaacctcg cgcatacagc 3360

cgggcagtga cgtcatcgtc tgcgcggaaa tggacgaaca gtggggctat gtcggggcta 3420

aatcgcgcca gcgctggctg ttttacgcgt atgacagtct ccggaagacg gttgttgcgc 3480

acgtattcgg tgaacgcact atggcgacgc tggggcgtct tatgagcctg ctgtcaccct 3540

ttgacgtggt gatatggatg acggatggct ggccgctgta tgaatcccgc ctgaagggaa 3600

agctgcacgt aatcagcaag cgatatacgc agcgaattga gcggcataac ctgaatctga 3660

ggcagcacct ggcacggctg ggacggaagt cgctgtcgtt ctcaaaatcg gtggagctgc 3720

atgacaaagt catcgggcat tatctgaaca taaaacacta tcaataagtt ggagtcatta 3780

cccaaccagg aagggcagcc cacctatcaa ggtgtactgc cttccagacg aacgaagagc 3840

gattgaggaa aaggcggcgg cggccggcat gagcctgtcg gcctacctgc tggccgtcgg 3900

ccagggctac aaaatcacgg gcgtcgtgga ctatgagcac gtccgcgagc tggcccgcat 3960

caatggcgac ctgggccgcc tgggcggcct gctgaaactc tggctcaccg acgacccgcg 4020

cacggcgcgg ttcggtgatg ccacgatcct cgccctgctg gcgaagatcg aagagaagca 4080

ggacgagctt ggcaaggtca tgatgggcgt ggtccgcccg agggcagagc catgactttt 4140

ttagccgcta aaacggccgg ggggtgcgcg tgattgccaa gcacgtcccc atgcgctcca 4200

tcaagaagag cgacttcgcg gagctggtat tcgtgcaggg caagattcgg aataccaagt 4260

acgagaagga cggccagacg gtctacggga ccgacttcat tgccgataag gtggattatc 4320

tggacaccaa ggcaccaggc gggtcaaatc aggaataagg gcacattgcc ccggcgtgag 4380

tcggggcaat cccgcaagga gggtgaatga atcggacgtt tgaccggaag gcatacaggc 4440

aagaactgat cgacgcgggg ttttccgccg aggatgccga aaccatcgca agccgcaccg 4500

tcatgcgtgc gccccgcgaa accttccagt ccgtcggctc gatggtccag caagctacgg 4560

ccaagatcga gcgcgacagc gtgcaactgg ctccccctgc cctgcccgcg ccatcggccg 4620

ccgtggagcg ttcgcgtcgt ctcgaacagg aggcggcagg tttggcgaag tcgatgacca 4680

tcgacacgcg aggaactatg acgaccaaga agcgaaaaac cgccggcgag gacctggcaa 4740

aacaggtcag cgaggccaag caggccgcgt tgctgaaaca cacgaagcag cagatcaagg 4800

aaatgcagct ttccttgttc gatattgcgc cgtggccgga cacgatgcga gcgatgccaa 4860

acgacacggc ccgctctgcc ctgttcacca cgcgcaacaa gaaaatcccg cgcgaggcgc 4920

tgcaaaacaa ggtcattttc cacgtcaaca aggacgtgaa gatcacctac accggcgtcg 4980

agctgcgggc cgacgatgac gaactggtgt ggcagcaggt gttggagtac gcgaagcgca 5040

cccctatcgg cgagccgatc accttcacgt tctacgagct ttgccaggac ctgggctggt 5100
cgatcaatgg ccggtattac acgaaggccg aggaatgcct gtcgcgccta caggcgacgg 5160
cgatgggctt cacgtccgac cgcgttgggc acctggaatc ggtgtcgctg ctgcaccgct 5220
tccgcgtcct ggaccgtggc aagaaaacgt cccgttgcca ggtcctgatc gacgaggaaa 5280
tcgtcgtgct gtttgctggc gaccactaca cgaaattcat atgggagaag taccgcaagc 5340
tgtcgccgac ggcccgacgg atgttcgact atttcagctc gcaccgggag ccgtacccgc 5400
tcaagctgga aaccttccgc ctcatgtgcg gatcggattc cacccgcgtg aagaagtggc 5460
gcgagcaggt cggcgaagcc tgcgaagagt tgcgaggcag cggcctggtg gaacacgcct 5520
gggtcaatga tgacctggtg cattgcaaac gctagggcct tgtggggtca gttccggctg 5580
ggggttcagc agccagcgct ttactggcat ttcaggaaca agcgggcact gctcgacgca 5640
cttgcttcgc tcagtatcgc tcgggacgca cggcgcgctc tacgaactgc cgataaacag 5700
aggattaaaa ttgacaattg tgattaaggc tcagattcga cggcttggag cggccgacgt 5760
gcaggatttc cgcgagatcc gattgtcggc cctgaagaaa gctccagaga tgttcgggtc 5820
cgtttacgag cacgaggaga aaaagcccat ggaggcgttc gctgaacggt tgcgagatgc 5880
cgtggcattc ggcgcctaca tcgacggcga gatcattggg ctgtcggtct tcaaacagga 5940
ggacggcccc aaggacgctc acaaggcgca tctgtccggc gttttcgtgg agcccgaaca 6000
gcgaggccga ggggtcgccg gtatgctgct gcgggcgttg ccggcgggtt tattgctcgt 6060
gatgatcgtc cgacagattc caacgggaat ctggtggatg cgcatcttca tcctcggcgc 6120
acttaatatt tcgctattct ggagcttgtt gtttatttcg gtctaccgcc tgccgggcgg 6180
ggtcgcggcg acggtaggcg ctgtgcagcc gctgatggtc gtgttcatct ctgccgctct 6240
gctaggtagc ccgatacgat tgatggcggt cctgggggct atttgcggaa ctgcgggcgt 6300
ggcgctgttg gtgttgacac caaacgcagc gctagatcct gtcggcgtcg cagcgggcct 6360
ggcgggggcg gtttccatgg cgttcggaac cgtgctgacc cgcaagtggc aacctcccgt 6420
gcctctgctc acctttaccg cctggcaact ggcggccgga ggacttctgc tcgttccagt 6480
agctttagtg tttgatccgc caatcccgat gcctacagga accaatgttc tcggcctggc 6540
gtggctcggc ctgatcggag cgggtttaac ctacttcctt tggttccggg ggatctcgcg 6600
actcgaacct acagttgttt ccttactggg ctttctcagc cgggatggcg ctaagaagct 6660
attgccgccg atcttcatat gcggtgtgaa ataccgcaca gatgcgtaag gagaaaatac 6720
cgcatcaggc gctcttccgc ttcctcgctc actgactcgc tgcgctcggt cgttcggctg 6780
cggcgagcgg tatcagctca ctcaaaggcg gtaatacggt tatccacaga atcaggggat 6840
aacgcaggaa agaacatgtg agcaaaaggc cagcaaaagg ccaggaaccg taaaaaggcc 6900

gcgttgctgg cgtttttcca taggctccgc ccccctgacg agcatcacaa aaatcgacgc 6960
tcaagtcaga ggtggcgaaa cccgacagga ctataaagat accaggcgtt tccccctgga 7020
agctccctcg tgcgctctcc tgttccgacc ctgccgctta ccggatacct gtccgccttt 7080
ctcccttcgg gaagcgtggc gctttctcaa tgctcacgct gtaggtatct cagttcggtg 7140
taggtcgttc gctccaagct gggctgtgtg cacgaacccc ccgttcagcc cgaccgctgc 7200
gccttatccg gtaactatcg tcttgagtcc aacccggtaa gacacgactt atcgccactg 7260
gcagcagcca ctggtaacag gattagcaga gcgaggtatg taggcggtgc tacagagttc 7320
ttgaagtggt ggcctaacta cggctacact agaaggacag tatttggtat ctgcgctctg 7380
ctgaagccag ttaccttcgg aaaaagagtt ggtagctctt gatccggcaa acaaaccacc 7440
gctggtagcg gtggtttttt tgtttgcaag cagcagatta cgcgcagaaa aaaaggatat 7500
caagaagatc ctttgatctt ttctacgggg tctgacgctc agtggaacga aaactcacgt 7560
taagggattt tggtcatgag attatcaaaa aggatcttca cctagatcct tttaaattaa 7620
aaatgaagtt ttaaatcaat ctaaagtata tatgagtaaa cttggtctga cagttaccaa 7680
tgcttaatca gtgaggcacc tatctcagcg atctgtctat ttcgttcatc catagttgcc 7740
tgactccccg tcgtgtagat aactacgata cgggagggct taccatctgg ccccagtgct 7800
gcaatgatac cgcgagaccc acgctcaccg gctccagatt tatcagcaat aaaccagcca 7860
gccggaaggg ccgagcgcag aagtggtcct gcaactttat ccgcctccat ccagtctatt 7920
aaacaagtgg cagcaacgga ttcgcaaacc tgtcacgcct tttgtgccaa aagccgcgcc 7980
aggtttgcga tccgctgtgc caggcgttag gcgtcatatg aagatttcgg tgatccctga 8040
gcaggtggcg gaaacattgg atgctgagaa ccatttcatt gttcgtgaag tgttcgatgt 8100
gcacctatcc gaccaaggct ttgaactatc taccagaagt gtgagcccct accggaagga 8160
ttacatctcg gatgatgact ctgatgaaga ctctgcttgc tatggcgcat tcatcgacca 8220
agagcttgtc gggaagattg aactcaactc aacatggaac gatctagcct ctatcgaaca 8280
cattgttgtg tcgcacacgc accgaggcaa aggagtcgcg cacagtctca tcgaatttgc 8340
gaaaaagtgg gcactaagca gacagctcct tggcatacga ttagagacac aaacgaacaa 8400
tgtacctgcc tgcaatttgt acgcaaaatg tggctttact ctcggcggca ttgacctgtt 8460
cacgtataaa actagacctc aagtctcgaa cgaaacagcg atgtactggt actggttctc 8520
gggagcacag gatgacgcct aacaattcat tcaagccgac accgcttcgc ggcgcggctt 8580
aattcaggag ttaaacatca tgagggaagc ggtgatcgcc gaagtatcga ctcaactatc 8640
agaggtagtt ggcgtcatcg agcgccatct cgaaccgacg ttgctggccg tacatttgta 8700
cggctccgca gtggatggcg gcctgaagcc acacagtgat attgatttgc tggttacggt 8760

gaccgtaagg cttgatgaaa caacgcggcg agctttgatc aacgaccttt tggaaacttc 8820
ggcttcccct ggagagagcg agattctccg cgctgtagaa gtcaccattg ttgtgcacga 8880
cgacatcatt ccgtggcgtt atccagctaa gcgcgaactg caatttggag aatggcagcg 8940
caatgacatt cttgcaggta tcttcgagcc agccacgatc gacattgatc tggctatctt 9000
gctgacaaaa gcaagagaac atagcgttgc cttggtaggt ccagcggcgg aggaactctt 9060
tgatccggtt cctgaacagg atctatttga ggcgctaaat gaaaccttaa cgctatggaa 9120
ctcgccgccc gactgggctg gcgatgagcg aaatgtagtg cttacgttgt cccgcatttg 9180
gtacagcgca gtaaccggca aaatcgcgcc gaaggatgtc gctgccgact gggcaatgga 9240
gcgcctgccg gcccagtatc agcccgtcat acttgaagct aggcaggctt atcttggaca 9300
agaagatcgc ttggcctcgc gcgcagatca gttggaagaa tttgttcact acgtgaaagg 9360
cgagatcacc aaggtagtcg gcaaataatg tctaacaatt cgttcaagcc gacgccgctt 9420
cgcggcgcgg cttaactcaa gcgttagaga gctggggaag actatgcgcg atctgttgaa 9480
ggtggttcta agcctcgtac ttgcgatggc atcggggcag gcacttgctg acctgccaat 9540
tgttttagtg gatgaagctc gtcttcccta tgactactcc ccatccaact acgacatttc 9600
tccaagcaac tacgacaact ccataagcaa ttacgacaat agtccatcaa attacgacaa 9660
ctctgagagc aactacgata atagttcatc caattacgac aatagtcgca acggaaatcg 9720
taggcttata tatagcgcaa atgggtctcg cactttcgcc ggctactacg tcattgccaa 9780
caatgggaca acgaacttct tttccacatc tggcaaaagg atgttctaca ccccaaaagg 9840
ggggcgcggc gtctatggcg gcaaagatgg gagcttctgc ggggcattgg tcgtcataaa 9900
tggccaattt tcgcttgccc tgacagataa cggcctgaag atcatgtatc taagcaacta 9960
gcctgctctc taataaaatg ttaggagctt ggctgccatt tttggggtga ggccgttcgc 10020
ggccgagggg cgcagcccct gggggatgg gaggcccgcg ttagcgggcc gggagggttc 10080
gagaaggggg ggcacccccc ttcggcgtgc gcggtcacgc gccagggcgc agccctggtt 10140
aaaaacaagg tttataaata ttggtttaaa agcaggttaa aagacaggtt agcggtggcc 10200
gaaaaacggg cggaaaccct tgcaaatgct ggattttctg cctgtggaca gcccctcaaa 10260
tgtcaatagg tgcgcccctc atctgtcagc actctgcccc tcaagtgtca aggatcgcgc 10320
ccctcatctg tcagtagtcg cgcccctcaa gtgtcaatac cgcagggcac ttatccccag 10380
gcttgtccac atcatctgtg ggaaactcgc gtaaaatcag gcgttttcgc cgatttgcga 10440
ggctggccag ctccacgtcg ccggccgaaa tcgagcctgc ccctcatctg tcaacgccgc 10500
gccgggtgag tcggcccctc aagtgtcaac gtccgcccct catctgtcag tgagggccaa 10560
gttttccgcg aggtatccac aacgccggcg gccggccgcg gtgtctcgca cacggcttcg 10620

acggcgtttc tggcgcgttt gcagggccat agacggccgc cagcccagcg gcgagggcaa 10680
ccagcccggt gagcgtcgga aagggtcgac atcttgctgc gttcggatat tttcgtggag 10740
ttcccgccac agacccggat tgaaggcgag atccagcaac tcgcgccaga tcatcctgtg 10800
acggaacttt ggcgcgtgat gactggccag gacgtcggcc gaaagagcga caagcagatc 10860
acgattttcg acagcgtcgg atttgcgatc gaggattttt cggcgctgcg ctacgtccgc 10920
gaccgcgttg agggatcaag ccacagcagc ccactcgacc ttctagccga cccagacgag 10980
ccaagggatc tttttggaat gctgctccgt cgtcaggctt tccgacgttt gggtggttga 11040
acagaagtca ttatcgtacg gaatgccagc actcccgagg ggaaccctgt ggttggcatg 11100
cacatacaaa tggacgaacg gataaacctt ttcacgccct tttaaatatc cgttattcta 11160
ataaacgctc ttttctctta ggtttacccg ccaatatatc ctgtcaaaca ctgatagttt 11220
aaactgaagg cgggaaacga caatctgatc atgagcggag aattaaggga gtcacgttat 11280
gacccccgcc gatgacgcgg gacaagccgt tttacgtttg gaactgacag aaccgcaacg 11340
attgaaggag ccactcagcc ccaatacgca aaccgcctct ccccgcgcgt tggccgattc 11400
attaatgcag ctggcacgac aggtttcccg actggaaagc gggcagtgag cgcaacgcaa 11460
ttaatgtgag ttagctcact cattaggcac cccaggcttt acactttatg cttccggctc 11520
gtatgttgtg tggaattgtg agcggataac aatttcacac aggaaacagc tatgaccatg 11580
attacgccaa gctatttagg tgacactata gaatactcaa gctatgcatc caacgcgttg 11640
ggagctctcc catatcgacc tgcaggcggc cgctcgacga attaattcca atcccacaaa 11700
aatctgagct taacagcaca gttgctcctc tcagagcaga atcgggtatt caacaccctc 11760
atatcaacta ctacgttgtg tataacggtc cacatgccgg tatatacgat gactggggtt 11820
gtacaaaggc ggcaacaaac ggcgttcccg gagttgcaca caagaaattt gccactatta 11880
cagaggcaag agcagcagct gacgcgtaca caacaagtca gcaaacagac aggttgaact 11940
tcatccccaa aggagaagct caactcaagc ccaagagctt tgctaaggcc ctaacaagcc 12000
caccaaagca aaaagcccac tggctcacgc taggaaccaa aaggcccagc agtgatccag 12060
ccccaaaaga gatctccttt gccccggaga ttacaatgga cgatttcctc tatctttacg 12120
atctaggaag gaagttcgaa ggtgaaggtg acgacactat gttcaccact gataatgaga 12180
aggttagcct cttcaatttc agaaagaatg ctgacccaca gatggttaga gaggcctacg 12240
cagcaggtct catcaagacg atctacccga gtaacaatct ccaggagatc aaataccttc 12300
ccaagaaggt taaagatgca gtcaaaagat tcaggactaa ttgcatcaag aacacagaga 12360
aagacatatt tctcaagatc agaagtacta ttccagtatg gacgattcaa ggcttgcttc 12420
ataaaccaag gcaagtaata gagattggag tctctaaaaa ggtagttcct actgaatcta 12480

aggccatgca tggagtctaa gattcaaatc gaggatctaa cagaactcgc cgtgaagact 12540
ggcgaacagt tcatacagag tcttttacga ctcaatgaca agaagaaaat cttcgtcaac 12600
atggtggagc acgacactct ggtctactcc aaaaatgtca aagatacagt ctcagaagac 12660
caaagggcta ttgagacttt tcaacaaagg ataatttcgg gaaacctcct cggattccat 12720
tgcccagcta tctgtcactt catcgaaagg acagtagaaa aggaaggtgg ctcctacaaa 12780
tgccatcatt gcgataaagg aaaggctatc attcaagatc tctctgccga cagtggtccc 12840
aaagatggac ccccacccac gaggagcatc gtggaaaaag aagacgttcc aaccacgtct 12900
tcaaagcaag tggattgatg tgacatctcc actgacgtaa gggatgacgc acaatcccac 12960
tatccttcgc aagacccttc ctctatataa ggaagttcat ttcatttgga gaggacacgc 13020
tcgagacaag tttgtacaaa aaagctgaac gagaaacgta aaatgatata aatatcaata 13080
tattaaatta gattttgcat aaaaaacaga ctacataata ctgtaaaaca caacatatcc 13140
agtcactatg aatcaactac ttagatggta ttagtgacct gtagtcgacc gacagccttc 13200
caaatgttct tcgggtgatg ctgccaactt agtcgaccga cagccttcca aatgttcttc 13260
tcaaacggaa tcgtcgtatc cagcctactc gctattgtcc tcaatgccgt attaaatcat 13320
aaaaagaaat aagaaaaaga ggtgcgagcc tcttttttgt gtgacaaaat aaaaacatct 13380
acctattcat atacgctagt gtcatagtcc tgaaaatcat ctgcatcaag aacaatttca 13440
caactcttat acttttctct tacaagtcgt tcggcttcat ctggattttc agcctctata 13500
cttactaaac gtgataaagt ttctgtaatt tctactgtat cgacctgcag actggctgtg 13560
tataagggag cctgacattt atattcccca gaacatcagg ttaatggcgt ttttgatgtc 13620
attttcgcgg tggctgagat cagccacttc ttccccgata acggagaccg gcacactggc 13680
catatcggtg gtcatcatgc gccagctttc atccccgata tgcaccaccg ggtaaagttc 13740
acgggagact ttatctgaca gcagacgtgc actggccagg gggatcacca tccgtcgccc 13800
gggcgtgtca ataatatcac tctgtacatc cacaaacaga cgataacggc tctctctttt 13860
ataggtgtaa accttaaact gcatttcacc agtccctgtt ctcgtcagca aagagccgt 13920
tcatttcaat aaaccgggcg acctcagcca tcccttcctg attttccgct ttccagcgtt 13980
cggcacgcag acgacgggct tcattctgca tggttgtgct taccagaccg gagatattga 14040
catcatatat gccttgagca actgatagct gtcgctgtca actgtcactg taatacgctg 14100
cttcatagca cacctctttt tgacatactt cgggtagtgc cgatcaacgt ctcattttcg 14160
ccaaaagttg gcccagggct tcccggtatc aacagggaca ccaggattta tttattctgc 14220
gaagtgatct tccgtcacag gtatttattc ggcgcaaagt gcgtcgggtg atgctgccaa 14280
cttagtcgac tacaggtcac taataccatc taagtagttg attcatagtg actggatatg 14340

ttgtgtttta cagtattatg tagtctgttt tttatgcaaa atctaattta atatattgat 14400
atttatatca ttttacgttt ctcgttcagc tttcttgtac aaagtggtct cgaggaattc 14460
ggtaccccag cttggtaagg aaataattat tttctttttt ccttttagta taaaatagtt 14520
aagtgatgtt aattagtatg attataataa tatagttgtt ataattgtga aaaaataatt 14580
tataaatata ttgtttacat aaacaacata gtaatgtaaa aaaatatgac aagtgatgtg 14640
taagacgaag aagataaaag ttgagagtaa gtatattatt tttaatgaat ttgatcgaac 14700
atgtaagatg atatactagc attaatattt gttttaatca taatagtaat tctagctggt 14760
ttgatgaatt aaatatcaat gataaaatac tatagtaaaa ataagaataa ataaattaaa 14820
ataatatttt tttatgatta atagtttatt atataattaa atatctatac cattactaaa 14880
tattttagtt taaaagttaa taaatatttt gttagaaatt ccaatctgct tgtaatttat 14940
caataaacaa aatattaaat aacaagctaa agtaacaaat aatatcaaac taatagaaac 15000
agtaatctaa tgtaacaaaa cataatctaa tgctaatata acaaagcgca agatctatca 15060
ttttatatag tattattttc aatcaacatt cttattaatt tctaaataat acttgtagtt 15120
ttattaactt ctaaatggat tgactattaa ttaaatgaat tagtcgaaca tgaataaaca 15180
aggtaacatg atagatcatg tcattgtgtt atcattgatc ttacatttgg attgattaca 15240
gttgggaagc tgggttcgaa atcgataagc ttggatcctc tagaccactt tgtacaagaa 15300
agctgaacga gaaacgtaaa atgatataaa tatcaatata ttaaattaga ttttgcataa 15360
aaaacagact acataatact gtaaaacaca acatatccag tcactatgaa tcaactactt 15420
agatggtatt agtgacctgt agtcgactaa gttggcagca tcacccgacg cactttgcgc 15480
cgaataaata cctgtgacgg aagatcactt cgcagaataa ataaatcctg gtgtccctgt 15540
tgataccggg aagccctggg ccaacttttg gcgaaaatga gacgttgatc ggatttcaca 15600
actcttatac ttttctctta caagtcgttc ggcttcatct ggattttcag cctctatact 15660
tactaaacgt gataaagttt ctgtaatttc tactgtatcg acctgcagac tggctgtgta 15720
taagggagcc tgacatttat attccccaga acatcaggtt aatggcgttt ttgatgtcat 15780
tttcgcggtg gctgagatca gccacttctt ccccgataac ggagaccggc acactggcca 15840
tatcggtggt catcatgcgc cagctttcat ccccgatatg caccaccggg taaagttcac 15900
gggagacttt atctgacagc agacgtgcac tggccagggg gatcaccatc cgtcgcccgg 15960
gcgtgtcaat aatatcactc tgtacatcca caaacagacg ataacggctc tctcttttat 16020
aggtgtaaac cttaaactgc atttcaccag tccctgttct cgtcagcaaa agagccgttc 16080
atttcaataa accgggcgac ctcagccatc ccttcctgat tttccgcttt ccagcgttcg 16140
gcacgcagac gacgggcttc attctgcatg gttgtgctta ccagaccgga gatattgaca 16200

tcatatatgc cttgagcaac tgatagctgt cgctgtcaac tgtcactgta atacgctgct 16260

tcatagcaca cctctttttg acatacttct gttcttgatg cagatgattt tcaggactat 16320

gacactagcg tatatgaata ggtagatgtt tttattttgt cacacaaaaa agaggctcgc 16380

acctcttttt cttatttctt tttatgattt aatacggcat tgaggacaat agcgagtagg 16440

ctggatacga cgattccgtt tgagaagaac atttggaagg ctgtcggtcg actaagttgg 16500

cagcatcacc cgaagaacat ttggaaggct gtcggtcgac tacaggtcac taataccatc 16560

taagtagttg attcatagtg actggatatg ttgtgtttta cagtattatg tagtctgttt 16620

tttatgcaaa atctaattta atatattgat atttatatca ttttacgttt ctcgttcagc 16680

tttttttgtac aaacttgtct agagtcctgc tttaatgaga tatgcgagac gcctatgatc 16740

gcatgatatt tgctttcaat tctgttgtgc acgttgtaaa aaacctgagc atgtgtagct 16800

cagatcctta ccgccggttt cggttcattc taatgaatat atcacccgtt actatcgtat 16860

ttttatgaat aatattctcc gttcaattta ctgattgtac cctactactt atatgtacaa 16920

tattaaaatg aaaacaatat attgtgctga ataggtttat agcgacatct atgatagagc 16980

gccacaataa caaacaattg cgttttatta ttacaaatcc aattttaaaa aaagcggcag 17040

aaccggtcaa acctaaaaga ctgattacat aaatcttatt caaatttcaa aaggccccag 17100

gggctagtat ctacgacaca ccgagcggcg aactaataac gttcactgaa gggaactccg 17160

gttccccgcc ggcgcgcatg ggtgagattc cttgaagttg agtattggcc gtccgctcta 17220

ccgaaagtta cgggcaccat tcaacccggt ccagcacggc ggccgggtaa ccgacttgct 17280

gccccgagaa ttatgcagca tttttttggt gtatgtgggc cccaaatgaa gtgcaggtca 17340

aaccttgaca gtgacgacaa atcgttgggc gggtccaggg cgaattttgc gacaacatgt 17400

cgaggctcag caggacctgc aggcatgcaa gctagcttac tagtgatgca tattctatag 17460

tgtcacctaa atctgc 17476

<210> 25
<211> 17458
<212> DNA
<213> Artificial sequence

<220>
<223> acceptor vector pHELLSGATE11

<400> 25
ggccgcacta gtgatatccc gcggccatgg cggccgggag catgcgacgt cgggcccaat 60

tcgccctata gtgagtcgta ttacaattca ctggccgtcg ttttacaacg tcgtgactgg 120

gaaaaccctg gcgttaccca acttaatcgc cttgcagcac atcccccttt cgccagctgg 180

cgtaatagcg aagaggcccg caccgatcgc ccttcccaac agttgcgcag cctgaatggc 240

```
gaatggaaat tgtaaacgtt aatgggtttc tggagtttaa tgagctaagc acatacgtca     300
gaaaccatta ttgcgcgttc aaaagtcgcc taaggtcact atcagctagc aaatatttct     360
tgtcaaaaat gctccactga cgttccataa attcccctcg gtatccaatt agagtctcat     420
attcactctc aatccaaata atctgcaatg gcaattacct tatccgcaac ttctttacct     480
atttccgccc ggatccgggc aggttctccg gccgcttggg tggagaggct attcggctat     540
gactgggcac aacagacaat cggctgctct gatgccgccg tgttccggct gtcagcgcag     600
gggcgcccgg ttctttttgt caagaccgac ctgtccggtg ccctgaatga actgcaggac     660
gaggcagcgc ggctatcgtg gctggccacg acgggcgttc cttgcgcagc tgtgctcgac     720
gttgtcactg aagcgggaag ggactggctg ctattgggcg aagtgccggg gcaggatctc     780
ctgtcatctc accttgctcc tgccgagaaa gtatccatca tggctgatgc aatgcggcgg     840
ctgcatacgc ttgatccggc tacctgccca ttcgaccacc aagcgaaaca tcgcatcgag     900
cgagcacgta ctcggatgga agccggtctt gtcgatcagg atgatctgga cgaagagcat     960
caggggctcg cgccagccga actgttcgcc aggctcaagg cgcgcatgcc cgacggcgag    1020
gatctcgtcg tgacccatgg cgatgcctgc ttgccgaata tcatggtgga aaatggccgc    1080
ttttctggat tcatcgactg tggccggctg ggtgtggcgg accgctatca ggacatagcg    1140
ttggctaccc gtgatattgc tgaagagctt ggcggcgaat gggctgaccg cttcctcgtg    1200
ctttacggta tcgccgctcc cgattcgcag cgcatcgcct tctatcgcct tcttgacgag    1260
ttcttctgag cgggactctg gggttcgaaa tgaccgacca agcgacgccc aacctgccat    1320
cacgagattt cgattccacc gccgccttct atgaaaggtt gggcttcgga atcgttttcc    1380
gggacgccgg ctggatgatc ctccagcgcg gggatctcat gctggagttc ttcgcccacc    1440
ccgatccaac acttacgttt gcaacgtcca agagcaaata gaccacgaac gccggaaggt    1500
tgccgcagcg tgtggattgc gtctcaattc tctcttgcag gaatgcaatg atgaatatga    1560
tactgactat gaaactttga gggaatactg cctagcaccg tcacctcata acgtgcatca    1620
tgcatgccct gacaacatgg aacatcgcta tttttctgaa gaattatgct cgttggagga    1680
tgtcgcggca attgcagcta ttgccaacat cgaactaccc ctcacgcatg cattcatcaa    1740
tattattcat gcggggaaag gcaagattaa tccaactggc aaatcatcca gcgtgattgg    1800
taacttcagt tccagcgact tgattcgttt tggtgctacc cacgttttca ataaggacga    1860
gatggtggag taaagaagga gtgcgtcgaa gcagatcgtt caaacatttg gcaataaagt    1920
ttcttaagat tgaatcctgt tgccggtctt gcgatgatta tcatataatt tctgttgaat    1980
tacgttaagc atgtaataat taacatgtaa tgcatgacgt tatttatgag atgggttttt    2040
atgattagag tcccgcaatt atacatttaa tacgcgatag aaaacaaaat atagcgcgca    2100
```

aactaggata aattatcgcg cgcggtgtca tctatgttac tagatcgaat taattccagg 2160
cggtgaaggg caatcagctg ttgcccgtct cactggtgaa aagaaaaacc accccagtac 2220
attaaaaacg tccgcaatgt gttattaagt tgtctaagcg tcaatttgtt tacaccacaa 2280
tatatcctgc caccagccag ccaacagctc cccgaccggc agctcggcac aaaatcacca 2340
ctcgatacag gcagcccatc agtccgggac ggcgtcagcg ggagagccgt tgtaaggcgg 2400
cagactttgc tcatgttacc gatgctattc ggaagaacgg caactaagct gccgggtttg 2460
aaacacggat gatctcgcgg agggtagcat gttgattgta acgatgacag agcgttgctg 2520
cctgtgatca aatatcatct ccctcgcaga gatccgaatt atcagccttc ttattcattt 2580
ctcgcttaac cgtgacaggc tgtcgatctt gagaactatg ccgacataat aggaaatcgc 2640
tggataaagc cgctgaggaa gctgagtggc gctatttctt tagaagtgaa cgttgacgat 2700
gtcgacggat cttttccgct gcataaccct gcttcggggt cattatagcg atttttcgg 2760
tatatccatc ctttttcgca cgatatacag gattttgcca aagggttcgt gtagactttc 2820
cttggtgtat ccaacggcgt cagccgggca ggataggtga agtaggccca cccgcgagcg 2880
ggtgttcctt cttcactgtc ccttattcgc acctggcggt gctcaacggg aatcctgctc 2940
tgcgaggctg gccggctacc gccggcgtaa cagatgaggg caagcggatg gctgatgaaa 3000
ccaagccaac caggggtgat gctgccaact tactgattta gtgtatgatg gtgtttttga 3060
ggtgctccag tggcttctgt ttctatcagc tgtccctcct gttcagctac tgacggggtg 3120
gtgcgtaacg gcaaaagcac cgccggacat cagcgctatc tctgctctca ctgccgtaaa 3180
acatggcaac tgcagttcac ttacaccgct tctcaacccg gtacgcacca gaaaatcatt 3240
gatatggcca tgaatggcgt tggatgccgg gcaacagccc gcattatggg cgttggcctc 3300
aacacgattt tacgtcactt aaaaaactca ggccgcagtc ggtaacctcg cgcatacagc 3360
cgggcagtga cgtcatcgtc tgcgcggaaa tggacgaaca gtggggctat gtcggggcta 3420
aatcgcgcca gcgctggctg ttttacgcgt atgacagtct ccggaagacg gttgttgcgc 3480
acgtattcgg tgaacgcact atggcgacgc tggggcgtct tatgagcctg ctgtcaccct 3540
ttgacgtggt gatatggatg acggatggct ggccgctgta tgaatcccgc ctgaagggaa 3600
agctgcacgt aatcagcaag cgatatacgc agcgaattga gcggcataac ctgaatctga 3660
ggcagcacct ggcacggctg ggacggaagt cgctgtcgtt ctcaaaatcg gtggagctgc 3720
atgacaaagt catcgggcat tatctgaaca taaaacacta tcaataagtt ggagtcatta 3780
cccaaccagg aagggcagcc cacctatcaa ggtgtactgc cttccagacg aacgaagagc 3840
gattgaggaa aaggcggcgg cggccggcat gagcctgtcg gcctacctgc tggccgtcgg 3900
ccagggctac aaaatcacgg gcgtcgtgga ctatgagcac gtccgcgagc tggcccgcat 3960

caatggcgac ctgggccgcc tgggcggcct gctgaaactc tggctcaccg acgacccgcg 4020
cacggcgcgg ttcggtgatg ccacgatcct cgccctgctg gcgaagatcg aagagaagca 4080
ggacgagctt ggcaaggtca tgatgggcgt ggtccgcccg agggcagagc catgactttt 4140
ttagccgcta aaacggccgg ggggtgcgcg tgattgccaa gcacgtcccc atgcgctcca 4200
tcaagaagag cgacttcgcg gagctggtat tcgtgcaggg caagattcgg aataccaagt 4260
acgagaagga cggccagacg gtctacggga ccgacttcat tgccgataag gtggattatc 4320
tggacaccaa ggcaccaggc gggtcaaatc aggaataagg gcacattgcc ccggcgtgag 4380
tcggggcaat cccgcaagga gggtgaatga atcggacgtt tgaccggaag gcatacaggc 4440
aagaactgat cgacgcgggg ttttccgccg aggatgccga aaccatcgca agccgcaccg 4500
tcatgcgtgc gccccgcgaa accttccagt ccgtcggctc gatggtccag caagctacgg 4560
ccaagatcga gcgcgacagc gtgcaactgg ctccccctgc cctgcccgcg ccatcggccg 4620
ccgtggagcg ttcgcgtcgt ctcgaacagg aggcggcagg tttggcgaag tcgatgacca 4680
tcgacacgcg aggaactatg acgaccaaga agcgaaaaac cgccggcgag gacctggcaa 4740
aacaggtcag cgaggccaag caggccgcgt tgctgaaaca cacgaagcag cagatcaagg 4800
aaatgcagct ttccttgttc gatattgcgc cgtggccgga cacgatgcga gcgatgccaa 4860
acgacacggc ccgctctgcc ctgttcacca cgcgcaacaa gaaaatcccg cgcgaggcgc 4920
tgcaaaacaa ggtcattttc cacgtcaaca aggacgtgaa gatcacctac accggcgtcg 4980
agctgcgggc cgacgatgac gaactggtgt ggcagcaggt gttggagtac gcgaagcgca 5040
cccctatcgg cgagccgatc accttcacgt tctacgagct ttgccaggac ctgggctggt 5100
cgatcaatgg ccggtattac acgaaggccg aggaatgcct gtcgcgccta caggcgacgg 5160
cgatgggctt cacgtccgac cgcgttgggc acctggaatc ggtgtcgctg ctgcaccgct 5220
tccgcgtcct ggaccgtggc aagaaaacgt cccgttgcca ggtcctgatc gacgaggaaa 5280
tcgtcgtgct gtttgctggc gaccactaca cgaaattcat atgggagaag taccgcaagc 5340
tgtcgccgac ggcccgacgg atgttcgact atttcagctc gcaccgggag ccgtacccgc 5400
tcaagctgga aaccttccgc ctcatgtgcg gatcggattc cacccgcgtg aagaagtggc 5460
gcgagcaggt cggcgaagcc tgcgaagagt tgcgaggcag cggcctggtg gaacacgcct 5520
gggtcaatga tgacctggtg cattgcaaac gctagggcct tgtggggtca gttccggctg 5580
ggggttcagc agccagcgct ttactggcat ttcaggaaca agcgggcact gctcgacgca 5640
cttgcttcgc tcagtatcgc tcgggacgca cggcgcgctc tacgaactgc cgataaacag 5700
aggattaaaa ttgacaattg tgattaaggc tcagattcga cggcttggag cggccgacgt 5760
gcaggatttc cgcgagatcc gattgtcggc cctgaagaaa gctccagaga tgttcgggtc 5820

cgtttacgag cacgaggaga aaaagcccat ggaggcgttc gctgaacggt tgcgagatgc 5880
cgtggcattc ggcgcctaca tcgacggcga gatcattggg ctgtcggtct tcaaacagga 5940
ggacggcccc aaggacgctc acaaggcgca tctgtccggc gttttcgtgg agcccgaaca 6000
gcgaggccga ggggtcgccg gtatgctgct gcgggcgttg ccggcgggtt tattgctcgt 6060
gatgatcgtc cgacagattc caacgggaat ctggtggatg cgcatcttca tcctcggcgc 6120
acttaatatt tcgctattct ggagcttgtt gtttatttcg gtctaccgcc tgccgggcgg 6180
ggtcgcggcg acggtaggcg ctgtgcagcc gctgatggtc gtgttcatct ctgccgctct 6240
gctaggtagc ccgatacgat tgatggcggt cctgggggct atttgcggaa ctgcgggcgt 6300
ggcgctgttg gtgttgacac caaacgcagc gctagatcct gtcggcgtcg cagcgggcct 6360
ggcgggggcg gtttccatgg cgttcggaac cgtgctgacc cgcaagtggc aacctcccgt 6420
gcctctgctc acctttaccg cctggcaact ggcggccgga ggacttctgc tcgttccagt 6480
agctttagtg tttgatccgc caatcccgat gcctacagga accaatgttc tcggcctggc 6540
gtggctcggc ctgatcggag cgggtttaac ctacttcctt tggttccggg ggatctcgcg 6600
actcgaacct acagttgttt ccttactggg ctttctcagc cgggatggcg ctaagaagct 6660
attgccgccg atcttcatat gcggtgtgaa ataccgcaca gatgcgtaag gagaaaatac 6720
cgcatcaggc gctcttccgc ttcctcgctc actgactcgc tgcgctcggt cgttcggctg 6780
cggcgagcgg tatcagctca ctcaaaggcg gtaatacggt tatccacaga atcaggggat 6840
aacgcaggaa agaacatgtg agcaaaaggc cagcaaaagg ccaggaaccg taaaaaggcc 6900
gcgttgctgg cgtttttcca taggctccgc ccccctgacg agcatcacaa aaatcgacgc 6960
tcaagtcaga ggtggcgaaa cccgacagga ctataaagat accaggcgtt tccccctgga 7020
agctccctcg tgcgctctcc tgttccgacc ctgccgctta ccggatacct gtccgccttt 7080
ctcccttcgg gaagcgtggc gctttctcaa tgctcacgct gtaggtatct cagttcggtg 7140
taggtcgttc gctccaagct gggctgtgtg cacgaacccc ccgttcagcc cgaccgctgc 7200
gccttatccg gtaactatcg tcttgagtcc aacccggtaa gacacgactt atcgccactg 7260
gcagcagcca ctggtaacag gattagcaga gcgaggtatg taggcggtgc tacagagttc 7320
ttgaagtggt ggcctaacta cggctacact agaaggacag tatttggtat ctgcgctctg 7380
ctgaagccag ttaccttcgg aaaaagagtt ggtagctctt gatccggcaa acaaaccacc 7440
gctggtagcg gtggtttttt tgtttgcaag cagcagatta cgcgcagaaa aaaaggatat 7500
caagaagatc ctttgatctt ttctacgggg tctgacgctc agtggaacga aaactcacgt 7560
taagggattt tggtcatgag attatcaaaa aggatcttca cctagatcct tttaaattaa 7620
aaatgaagtt ttaaatcaat ctaaagtata tatgagtaaa cttggtctga cagttaccaa 7680

tgcttaatca gtgaggcacc tatctcagcg atctgtctat ttcgttcatc catagttgcc 7740
tgactccccg tcgtgtagat aactacgata cgggagggct taccatctgg ccccagtgct 7800
gcaatgatac cgcgagaccc acgctcaccg gctccagatt tatcagcaat aaaccagcca 7860
gccggaaggg ccgagcgcag aagtggtcct gcaactttat ccgcctccat ccagtctatt 7920
aaacaagtgg cagcaacgga ttcgcaaacc tgtcacgcct tttgtgccaa aagccgcgcc 7980
aggtttgcga tccgctgtgc caggcgttag gcgtcatatg aagatttcgg tgatccctga 8040
gcaggtggcg gaaacattgg atgctgagaa ccatttcatt gttcgtgaag tgttcgatgt 8100
gcacctatcc gaccaaggct ttgaactatc taccagaagt gtgagcccct accggaagga 8160
ttacatctcg gatgatgact ctgatgaaga ctctgcttgc tatggcgcat tcatcgacca 8220
agagcttgtc gggaagattg aactcaactc aacatggaac gatctagcct ctatcgaaca 8280
cattgttgtg tcgcacacgc accgaggcaa aggagtcgcg cacagtctca tcgaatttgc 8340
gaaaaagtgg gcactaagca gacagctcct tggcatacga ttagagacac aaacgaacaa 8400
tgtacctgcc tgcaatttgt acgcaaaatg tggctttact ctcggcggca ttgacctgtt 8460
cacgtataaa actagacctc aagtctcgaa cgaaacagcg atgtactggt actggttctc 8520
gggagcacag gatgacgcct aacaattcat tcaagccgac accgcttcgc ggcgcggctt 8580
aattcaggag ttaaacatca tgagggaagc ggtgatcgcc gaagtatcga ctcaactatc 8640
agaggtagtt ggcgtcatcg agcgccatct cgaaccgacg ttgctggccg tacatttgta 8700
cggctccgca gtggatggcg gcctgaagcc acacagtgat attgatttgc tggttacggt 8760
gaccgtaagg cttgatgaaa caacgcggcg agctttgatc aacgaccttt tggaaacttc 8820
ggcttcccct ggagagagcg agattctccg cgctgtagaa gtcaccattg ttgtgcacga 8880
cgacatcatt ccgtggcgtt atccagctaa gcgcgaactg caatttggag aatggcagcg 8940
caatgacatt cttgcaggta tcttcgagcc agccacgatc gacattgatc tggctatctt 9000
gctgacaaaa gcaagagaac atagcgttgc cttggtaggt ccagcggcgg aggaactctt 9060
tgatccggtt cctgaacagg atctatttga ggcgctaaat gaaaccttaa cgctatggaa 9120
ctcgccgccc gactgggctg gcgatgagcg aaatgtagtg cttacgttgt cccgcatttg 9180
gtacagcgca gtaaccggca aaatcgcgcc gaaggatgtc gctgccgact gggcaatgga 9240
gcgcctgccg gcccagtatc agcccgtcat acttgaagct aggcaggctt atcttggaca 9300
agaagatcgc ttggcctcgc gcgcagatca gttggaagaa tttgttcact acgtgaaagg 9360
cgagatcacc aaggtagtcg gcaaataatg tctaacaatt cgttcaagcc gacgccgctt 9420
cgcggcgcgg cttaactcaa gcgttagaga gctggggaag actatgcgcg atctgttgaa 9480
ggtggttcta agcctcgtac ttgcgatggc atcggggcag gcacttgctg acctgccaat 9540

tgttttagtg gatgaagctc gtcttcccta tgactactcc ccatccaact acgacatttc 9600
tccaagcaac tacgacaact ccataagcaa ttacgacaat agtccatcaa attacgacaa 9660
ctctgagagc aactacgata atagttcatc caattacgac aatagtcgca acggaaatcg 9720
taggcttata tatagcgcaa atgggtctcg cactttcgcc ggctactacg tcattgccaa 9780
caatgggaca acgaacttct tttccacatc tggcaaaagg atgttctaca ccccaaaagg 9840
ggggcgcggc gtctatggcg gcaaagatgg gagcttctgc ggggcattgg tcgtcataaa 9900
tggccaattt tcgcttgccc tgacagataa cggcctgaag atcatgtatc taagcaacta 9960
gcctgctctc taataaaatg ttaggagctt ggctgccatt tttggggtga ggccgttcgc 10020
ggccgagggg cgcagcccct gggggatgg gaggcccgcg ttagcgggcc gggagggttc 10080
gagaaggggg ggcacccccc ttcggcgtgc gcggtcacgc gccagggcgc agccctggtt 10140
aaaaacaagg tttataaata ttggtttaaa agcaggttaa aagacaggtt agcggtggcc 10200
gaaaaacggg cggaaaccct tgcaaatgct ggattttctg cctgtggaca gcccctcaaa 10260
tgtcaatagg tgcgcccctc atctgtcagc actctgcccc tcaagtgtca aggatcgcgc 10320
ccctcatctg tcagtagtcg cgcccctcaa gtgtcaatac cgcagggcac ttatccccag 10380
gcttgtccac atcatctgtg ggaaactcgc gtaaaatcag gcgttttcgc cgatttgcga 10440
ggctggccag ctccacgtcg ccggccgaaa tcgagcctgc ccctcatctg tcaacgccgc 10500
gccgggtgag tcggcccctc aagtgtcaac gtccgcccct catctgtcag tgagggccaa 10560
gttttccgcg aggtatccac aacgccggcg gccggccgcg gtgtctcgca cacggcttcg 10620
acggcgtttc tggcgcgttt gcagggccat agacggccgc cagcccagcg gcgagggcaa 10680
ccagcccggt gagcgtcgga aagggtcgac atcttgctgc gttcggatat ttcgtggag 10740
ttcccgccac agacccggat tgaaggcgag atccagcaac tcgcgccaga tcatcctgtg 10800
acggaacttt ggcgcgtgat gactggccag gacgtcggcc gaaagagcga caagcagatc 10860
acgattttcg acagcgtcgg atttgcgatc gaggattttt cggcgctgcg ctacgtccgc 10920
gaccgcgttg agggatcaag ccacagcagc ccactcgacc ttctagccga cccagacgag 10980
ccaagggatc tttttggaat gctgctccgt cgtcaggctt tccgacgttt gggtggttga 11040
acagaagtca ttatcgtacg gaatgccagc actcccgagg ggaaccctgt ggttggcatg 11100
cacatacaaa tggacgaacg gataaacctt ttcacgccct tttaaatatc cgttattcta 11160
ataaacgctc ttttctctta ggtttacccg ccaatatatc ctgtcaaaca ctgatagttt 11220
aaactgaagg cgggaaacga caatctgatc atgagcggag aattaaggga gtcacgttat 11280
gacccccgcc gatgacgcgg gacaagccgt tttacgtttg gaactgacag aaccgcaacg 11340
attgaaggag ccactcagcc ccaatacgca aaccgcctct ccccgcgcgt tggccgattc 11400

attaatgcag ctggcacgac aggtttcccg actggaaagc gggcagtgag cgcaacgcaa 11460
ttaatgtgag ttagctcact cattaggcac cccaggcttt acactttatg cttccggctc 11520
gtatgttgtg tggaattgtg agcggataac aatttcacac aggaaacagc tatgaccatg 11580
attacgccaa gctatttagg tgacactata gaatactcaa gctatgcatc caacgcgttg 11640
ggagctctcc catatcgacc tgcaggcggc cgctcgacga attaattcca atcccacaaa 11700
aatctgagct taacagcaca gttgctcctc tcagagcaga atcgggtatt caacaccctc 11760
atatcaacta ctacgttgtg tataacggtc cacatgccgg tatatacgat gactggggtt 11820
gtacaaaggc ggcaacaaac ggcgttcccg gagttgcaca caagaaattt gccactatta 11880
cagaggcaag agcagcagct gacgcgtaca caacaagtca gcaaacagac aggttgaact 11940
tcatccccaa aggagaagct caactcaagc ccaagagctt tgctaaggcc ctaacaagcc 12000
caccaaagca aaaagcccac tggctcacgc taggaaccaa aaggcccagc agtgatccag 12060
ccccaaaaga gatctccttt gccccggaga ttacaatgga cgatttcctc tatctttacg 12120
atctaggaag gaagttcgaa ggtgaaggtg acgacactat gttcaccact gataatgaga 12180
aggttagcct cttcaatttc agaaagaatg ctgacccaca gatggttaga gaggcctacg 12240
cagcaggtct catcaagacg atctacccga gtaacaatct ccaggagatc aaataccttc 12300
ccaagaaggt taaagatgca gtcaaaagat tcaggactaa ttgcatcaag aacacagaga 12360
aagacatatt tctcaagatc agaagtacta ttccagtatg gacgattcaa ggcttgcttc 12420
ataaaccaag gcaagtaata gagattggag tctctaaaaa ggtagttcct actgaatcta 12480
aggccatgca tggagtctaa gattcaaatc gaggatctaa cagaactcgc cgtgaagact 12540
ggcgaacagt tcatacagag tcttttacga ctcaatgaca agaagaaaat cttcgtcaac 12600
atggtggagc acgacactct ggtctactcc aaaaatgtca aagatacagt ctcagaagac 12660
caaagggcta ttgagacttt tcaacaaagg ataatttcgg gaaacctcct cggattccat 12720
tgcccagcta tctgtcactt catcgaaagg acagtagaaa aggaaggtgg ctcctacaaa 12780
tgccatcatt gcgataaagg aaaggctatc attcaagatc tctctgccga cagtggtccc 12840
aaagatggac ccccacccac gaggagcatc gtggaaaaag aagacgttcc aaccacgtct 12900
tcaaagcaag tggattgatg tgacatctcc actgacgtaa gggatgacgc acaatcccac 12960
tatccttcgc aagacccttc ctctatataa ggaagttcat ttcatttgga gaggacacgc 13020
tcgagacaag tttgtacaaa aaagctgaac gagaaacgta aaatgatata aatatcaata 13080
tattaaatta gattttgcat aaaaaacaga ctacataata ctgtaaaaca caacatatcc 13140
agtcactatg aatcaactac ttagatggta ttagtgacct gtagtcgacc gacagccttc 13200
caaatgttct tcgggtgatg ctgccaactt agtcgaccga cagccttcca aatgttcttc 13260

tcaaacggaa tcgtcgtatc cagcctactc gctattgtcc tcaatgccgt attaaatcat 13320
aaaaagaaat aagaaaaaga ggtgcgagcc tctttttgt gtgacaaaat aaaaacatct 13380
acctattcat atacgctagt gtcatagtcc tgaaaatcat ctgcatcaag aacaatttca 13440
caactcttat acttttctct tacaagtcgt tcggcttcat ctggattttc agcctctata 13500
cttactaaac gtgataaagt ttctgtaatt tctactgtat cgacctgcag actggctgtg 13560
tataagggag cctgacattt atattcccca gaacatcagg ttaatggcgt tttgatgtc 13620
attttcgcgg tggctgagat cagccacttc ttccccgata acggagaccg gcacactggc 13680
catatcggtg gtcatcatgc gccagctttc atccccgata tgcaccaccg ggtaaagttc 13740
acgggagact ttatctgaca gcagacgtgc actggccagg gggatcacca tccgtcgccc 13800
gggcgtgtca ataatatcac tctgtacatc cacaaacaga cgataacggc tctctctttt 13860
ataggtgtaa accttaaact gcatttcacc agtccctgtt ctcgtcagca aaagagccgt 13920
tcatttcaat aaaccgggcg acctcagcca tcccttcctg attttccgct ttccagcgtt 13980
cggcacgcag acgacgggct tcattctgca tggttgtgct taccagaccg gagatattga 14040
catcatatat gccttgagca actgatagct gtcgctgtca actgtcactg taatacgctg 14100
cttcatagca cacctctttt tgacatactt cgggtagtgc cgatcaacgt ctcattttcg 14160
ccaaaagttg gcccagggct tcccggtatc aacagggaca ccaggattta tttattctgc 14220
gaagtgatct tccgtcacag gtatttattc ggcgcaaagt gcgtcgggtg atgctgccaa 14280
cttagtcgac tacaggtcac taataccatc taagtagttg attcatagtg actggatatg 14340
ttgtgtttta cagtattatg tagtctgttt tttatgcaaa atctaattta atatattgat 14400
atttatatca ttttacgttt ctcgttcagc tttcttgtac aaagtggtct cgaggaattc 14460
ggtaccaact gtaaggaaat aattattttc ttttttcctt ttagtataaa atagttaagt 14520
gatgttaatt agtatgatta taataatata gttgttataa ttgtgaaaaa ataatttata 14580
aatatattgt ttacataaac aacatagtaa tgtaaaaaaa tatgacaagt gatgtgtaag 14640
acgaagaaga taaaagttga gagtaagtat attattttta atgaatttga tcgaacatgt 14700
aagatgatat actagcatta atatttgttt taatcataat agtaattcta gctggtttga 14760
tgaattaaat atcaatgata aaatactata gtaaaaataa gaataaataa attaaaataa 14820
tattttttta tgattaatag tttattatat aattaaatat ctataccatt actaaatatt 14880
ttagtttaaa agttaataaa tattttgtta gaaattccaa tctgcttgta atttatcaat 14940
aaacaaaata ttaaataaca agctaaagta acaaataata tcaaactaat agaaacagta 15000
atctaatgta acaaaacata atctaatgct aatataacaa agcgcaagat ctatcatttt 15060
atatagtatt attttcaatc aacattctta ttaatttcta aataatactt gtagttttat 15120

```
taacttctaa atggattgac tattaattaa atgaattagt cgaacatgaa taaacaaggt 15180
aacatgatag atcatgtcat tgtgttatca ttgatcttac atttggattg attacagtta 15240
cttaccttaa gcttggatcc tctagaccac tttgtacaag aaagctgaac gagaaacgta 15300
aaatgatata aatatcaata tattaaatta gattttgcat aaaaaacaga ctacataata 15360
ctgtaaaaca caacatatcc agtcactatg aatcaactac ttagatggta ttagtgacct 15420
gtagtcgact aagttggcag catcacccga cgcactttgc gccgaataaa tacctgtgac 15480
ggaagatcac ttcgcagaat aaataaatcc tggtgtccct gttgataccg ggaagccctg 15540
ggccaacttt tggcgaaaat gagacgttga tcggatttca caactcttat acttttctct 15600
tacaagtcgt tcggcttcat ctggattttc agcctctata cttactaaac gtgataaagt 15660
ttctgtaatt tctactgtat cgacctgcag actggctgtg tataagggag cctgacattt 15720
atattcccca gaacatcagg ttaatggcgt ttttgatgtc attttcgcgg tggctgagat 15780
cagccacttc ttccccgata acggagaccg gcacactggc catatcggtg gtcatcatgc 15840
gccagctttc atccccgata tgcaccaccg ggtaaagttc acgggagact ttatctgaca 15900
gcagacgtgc actggccagg gggatcacca tccgtcgccc gggcgtgtca ataatatcac 15960
tctgtacatc cacaaacaga cgataacggc tctctctttt ataggtgtaa accttaaact 16020
gcatttcacc agtccctgtt ctcgtcagca aaagagccgt tcatttcaat aaaccgggcg 16080
acctcagcca tcccttcctg attttccgct ttccagcgtt cggcacgcag acgacgggct 16140
tcattctgca tggttgtgct taccagaccg gagatattga catcatatat gccttgagca 16200
actgatagct gtcgctgtca actgtcactg taatacgctg cttcatagca cacctctttt 16260
tgacatactt ctgttcttga tgcagatgat tttcaggact atgacactag cgtatatgaa 16320
taggtagatg tttttatttt gtcacacaaa aaagaggctc gcacctcttt ttcttatttc 16380
tttttatgat ttaatacggc attgaggaca atagcgagta ggctggatac gacgattccg 16440
tttgagaaga acatttggaa ggctgtcggt cgactaagtt ggcagcatca cccgaagaac 16500
atttggaagg ctgtcggtcg actacaggtc actaatacca tctaagtagt tgattcatag 16560
tgactggata tgttgtgttt tacagtatta tgtagtctgt tttttatgca aaatctaatt 16620
taatatattg atatttatat cattttacgt ttctcgttca gctttttttgt acaaacttgt 16680
ctagagtcct gctttaatga gatatgcgag acgcctatga tcgcatgata tttgctttca 16740
attctgttgt gcacgttgta aaaaacctga gcatgtgtag ctcagatcct taccgccggt 16800
ttcggttcat tctaatgaat atatcacccg ttactatcgt atttttatga ataatattct 16860
ccgttcaatt tactgattgt accctactac ttatatgtac aatattaaaa tgaaaacaat 16920
atattgtgct gaataggttt atagcgacat ctatgataga gcgccacaat aacaaacaat 16980
```

tgcgttttat tattacaaat ccaattttaa aaaaagcggc agaaccggtc aaacctaaaa 17040
gactgattac ataaatctta ttcaaatttc aaaaggcccc aggggctagt atctacgaca 17100
caccgagcgg cgaactaata acgttcactg aagggaactc cggttccccg ccggcgcgca 17160
tgggtgagat tccttgaagt tgagtattgg ccgtccgctc taccgaaagt tacgggcacc 17220
attcaacccg gtccagcacg gcggccgggt aaccgacttg ctgccccgag aattatgcag 17280
catttttttg gtgtatgtgg gccccaaatg aagtgcaggt caaaccttga cagtgacgac 17340
aaatcgttgg gcgggtccag ggcgaatttt gcgacaacat gtcgaggctc agcaggacct 17400
gcaggcatgc aagctagctt actagtgatg catattctat agtgtcacct aaatctgc 17458

<210> 26
<211> 17681
<212> DNA
<213> Artificial sequence

<220>
<223> acceptor vector pHELLSGATE12

<400> 26
ggccgcacta gtgatatccc gcggccatgg cggccgggag catgcgacgt cgggcccaat 60
tcgccctata gtgagtcgta ttacaattca ctggccgtcg ttttacaacg tcgtgactgg 120
gaaaaccctg gcgttaccca acttaatcgc cttgcagcac atcccccttt cgccagctgg 180
cgtaatagcg aagaggcccg caccgatcgc ccttcccaac agttgcgcag cctgaatggc 240
gaatggaaat tgtaaacgtt aatgggtttc tggagtttaa tgagctaagc acatacgtca 300
gaaaccatta ttgcgcgttc aaaagtcgcc taaggtcact atcagctagc aaatatttct 360
tgtcaaaaat gctccactga cgttccataa attcccctcg gtatccaatt agagtctcat 420
attcactctc aatccaaata atctgcaatg gcaattacct tatccgcaac ttctttacct 480
atttccgccc ggatccgggc aggttctccg gccgcttggg tggagaggct attcggctat 540
gactgggcac aacagacaat cggctgctct gatgccgccg tgttccggct gtcagcgcag 600
gggcgcccgg ttctttttgt caagaccgac ctgtccggtg ccctgaatga actgcaggac 660
gaggcagcgc ggctatcgtg gctggccacg acgggcgttc cttgcgcagc tgtgctcgac 720
gttgtcactg aagcgggaag ggactggctg ctattgggcg aagtgccggg gcaggatctc 780
ctgtcatctc accttgctcc tgccgagaaa gtatccatca tggctgatgc aatgcggcgg 840
ctgcatacgc ttgatccggc tacctgccca ttcgaccacc aagcgaaaca tcgcatcgag 900
cgagcacgta ctcggatgga agccggtctt gtcgatcagg atgatctgga cgaagagcat 960
caggggctcg cgccagccga actgttcgcc aggctcaagg cgcgcatgcc cgacggcgag 1020
gatctcgtcg tgacccatgg cgatgcctgc ttgccgaata tcatggtgga aaatggccgc 1080

ttttctggat tcatcgactg tggccggctg ggtgtggcgg accgctatca ggacatagcg 1140
ttggctaccc gtgatattgc tgaagagctt ggcggcgaat gggctgaccg cttcctcgtg 1200
ctttacggta tcgccgctcc cgattcgcag cgcatcgcct tctatcgcct tcttgacgag 1260
ttcttctgag cgggactctg gggttcgaaa tgaccgacca agcgacgccc aacctgccat 1320
cacgagattt cgattccacc gccgccttct atgaaaggtt gggcttcgga atcgttttcc 1380
gggacgccgg ctggatgatc ctccagcgcg gggatctcat gctggagttc ttcgcccacc 1440
ccgatccaac acttacgttt gcaacgtcca agagcaaata gaccacgaac gccggaaggt 1500
tgccgcagcg tgtggattgc gtctcaattc tctcttgcag gaatgcaatg atgaatatga 1560
tactgactat gaaactttga gggaatactg cctagcaccg tcacctcata acgtgcatca 1620
tgcatgccct gacaacatgg aacatcgcta tttttctgaa gaattatgct cgttggagga 1680
tgtcgcggca attgcagcta ttgccaacat cgaactaccc ctcacgcatg cattcatcaa 1740
tattattcat gcgggggaaag gcaagattaa tccaactggc aaatcatcca gcgtgattgg 1800
taacttcagt tccagcgact tgattcgttt tggtgctacc cacgttttca ataaggacga 1860
gatggtggag taaagaagga gtgcgtcgaa gcagatcgtt caaacatttg gcaataaagt 1920
ttcttaagat tgaatcctgt tgccggtctt gcgatgatta tcatataatt tctgttgaat 1980
tacgttaagc atgtaataat taacatgtaa tgcatgacgt tatttatgag atgggttttt 2040
atgattagag tcccgcaatt atacatttaa tacgcgatag aaaacaaaat atagcgcgca 2100
aactaggata aattatcgcg cgcggtgtca tctatgttac tagatcgaat taattccagg 2160
cggtgaaggg caatcagctg ttgcccgtct cactggtgaa aagaaaaacc accccagtac 2220
attaaaaacg tccgcaatgt gttattaagt tgtctaagcg tcaatttgtt tacaccacaa 2280
tatatcctgc caccagccag ccaacagctc cccgaccggc agctcggcac aaaatcacca 2340
ctcgatacag gcagcccatc agtccgggac ggcgtcagcg ggagagccgt tgtaaggcgg 2400
cagactttgc tcatgttacc gatgctattc ggaagaacgg caactaagct gccgggtttg 2460
aaacacggat gatctcgcgg agggtagcat gttgattgta acgatgacag agcgttgctg 2520
cctgtgatca aatatcatct ccctcgcaga gatccgaatt atcagccttc ttattcattt 2580
ctcgcttaac cgtgacaggc tgtcgatctt gagaactatg ccgacataat aggaaatcgc 2640
tggataaagc cgctgaggaa gctgagtggc gctatttctt tagaagtgaa cgttgacgat 2700
gtcgacggat cttttccgct gcataaccct gcttcggggt cattatagcg atttttttcgg 2760
tatatccatc ctttttcgca cgatatacag gattttgcca aagggttcgt gtagactttc 2820
cttggtgtat ccaacggcgt cagccgggca ggataggtga agtaggccca cccgcgagcg 2880
ggtgttcctt cttcactgtc ccttattcgc acctggcggt gctcaacggg aatcctgctc 2940

tgcgaggctg gccggctacc gccggcgtaa cagatgaggg caagcggatg gctgatgaaa 3000
ccaagccaac caggggtgat gctgccaact tactgattta gtgtatgatg gtgtttttga 3060
ggtgctccag tggcttctgt ttctatcagc tgtccctcct gttcagctac tgacggggtg 3120
gtgcgtaacg gcaaaagcac cgccggacat cagcgctatc tctgctctca ctgccgtaaa 3180
acatggcaac tgcagttcac ttacaccgct tctcaacccg gtacgcacca gaaaatcatt 3240
gatatggcca tgaatggcgt tggatgccgg gcaacagccc gcattatggg cgttggcctc 3300
aacacgattt tacgtcactt aaaaaactca ggccgcagtc ggtaacctcg cgcatacagc 3360
cgggcagtga cgtcatcgtc tgcgcggaaa tggacgaaca gtggggctat gtcggggcta 3420
aatcgcgcca gcgctggctg ttttacgcgt atgacagtct ccggaagacg gttgttgcgc 3480
acgtattcgg tgaacgcact atggcgacgc tggggcgtct tatgagcctg ctgtcacccct 3540
ttgacgtggt gatatggatg acggatggct ggccgctgta tgaatcccgc ctgaagggaa 3600
agctgcacgt aatcagcaag cgatatacgc agcgaattga gcggcataac ctgaatctga 3660
ggcagcacct ggcacggctg ggacggaagt cgctgtcgtt ctcaaaatcg gtggagctgc 3720
atgacaaagt catcgggcat tatctgaaca taaaacacta tcaataagtt ggagtcatta 3780
cccaaccagg aagggcagcc cacctatcaa ggtgtactgc cttccagacg aacgaagagc 3840
gattgaggaa aaggcggcgg cggccggcat gagcctgtcg gcctacctgc tggccgtcgg 3900
ccagggctac aaaatcacgg gcgtcgtgga ctatgagcac gtccgcgagc tggcccgcat 3960
caatggcgac ctgggccgcc tgggcggcct gctgaaactc tggctcaccg acgacccgcg 4020
cacggcgcgg ttcggtgatg ccacgatcct cgccctgctg gcgaagatcg aagagaagca 4080
ggacgagctt ggcaaggtca tgatgggcgt ggtccgcccg agggcagagc catgactttt 4140
ttagccgcta aaacggccgg ggggtgcgcg tgattgccaa gcacgtcccc atgcgctcca 4200
tcaagaagag cgacttcgcg gagctggtat tcgtgcaggg caagattcgg aataccaagt 4260
acgagaagga cggccagacg gtctacggga ccgacttcat tgccgataag gtggattatc 4320
tggacaccaa ggcaccaggc gggtcaaatc aggaataagg gcacattgcc ccggcgtgag 4380
tcggggcaat cccgcaagga gggtgaatga atcggacgtt tgaccggaag gcatacaggc 4440
aagaactgat cgacgcgggg ttttccgccg aggatgccga aaccatcgca agccgcaccg 4500
tcatgcgtgc gccccgcgaa accttccagt ccgtcggctc gatggtccag caagctacgg 4560
ccaagatcga gcgcgacagc gtgcaactgg ctcccccctgc cctgcccgcg ccatcggccg 4620
ccgtggagcg ttcgcgtcgt ctcgaacagg aggcggcagg tttggcgaag tcgatgacca 4680
tcgacacgcg aggaactatg acgaccaaga agcgaaaaac cgccggcgag gacctggcaa 4740
aacaggtcag cgaggccaag caggccgcgt tgctgaaaca cacgaagcag cagatcaagg 4800

aaatgcagct ttccttgttc gatattgcgc cgtggccgga cacgatgcga gcgatgccaa 4860
acgacacggc ccgctctgcc ctgttcacca cgcgcaacaa gaaaatcccg cgcgaggcgc 4920
tgcaaaacaa ggtcattttc cacgtcaaca aggacgtgaa gatcacctac accggcgtcg 4980
agctgcgggc cgacgatgac gaactggtgt ggcagcaggt gttggagtac gcgaagcgca 5040
cccctatcgg cgagccgatc accttcacgt tctacgagct ttgccaggac ctgggctggt 5100
cgatcaatgg ccggtattac acgaaggccg aggaatgcct gtcgcgccta caggcgacgg 5160
cgatgggctt cacgtccgac cgcgttgggc acctggaatc ggtgtcgctg ctgcaccgct 5220
tccgcgtcct ggaccgtggc aagaaaacgt cccgttgcca ggtcctgatc gacgaggaaa 5280
tcgtcgtgct gtttgctggc gaccactaca cgaaattcat atgggagaag taccgcaagc 5340
tgtcgccgac ggcccgacgg atgttcgact atttcagctc gcaccgggag ccgtacccgc 5400
tcaagctgga aaccttccgc ctcatgtgcg gatcggattc cacccgcgtg aagaagtggc 5460
gcgagcaggt cggcgaagcc tgcgaagagt tgcgaggcag cggcctggtg gaacacgcct 5520
gggtcaatga tgacctggtg cattgcaaac gctagggcct tgtggggtca gttccggctg 5580
ggggttcagc agccagcgct ttactggcat ttcaggaaca agcgggcact gctcgacgca 5640
cttgcttcgc tcagtatcgc tcgggacgca cggcgcgctc tacgaactgc cgataaacag 5700
aggattaaaa ttgacaattg tgattaaggc tcagattcga cggcttggag cggccgacgt 5760
gcaggatttc cgcgagatcc gattgtcggc cctgaagaaa gctccagaga tgttcgggtc 5820
cgtttacgag cacgaggaga aaaagcccat ggaggcgttc gctgaacggt tgcgagatgc 5880
cgtggcattc ggcgcctaca tcgacggcga gatcattggg ctgtcggtct tcaaacagga 5940
ggacggcccc aaggacgctc acaaggcgca tctgtccggc gttttcgtgg agcccgaaca 6000
gcgaggccga ggggtcgccg gtatgctgct gcgggcgttg ccggcgggtt tattgctcgt 6060
gatgatcgtc cgacagattc caacgggaat ctggtggatg cgcatcttca tcctcggcgc 6120
acttaatatt tcgctattct ggagcttgtt gtttatttcg gtctaccgcc tgccgggcgg 6180
ggtcgcggcg acggtaggcg ctgtgcagcc gctgatggtc gtgttcatct ctgccgctct 6240
gctaggtagc ccgatacgat tgatggcggt cctgggggct atttgcggaa ctgcgggcgt 6300
ggcgctgttg gtgttgacac caaacgcagc gctagatcct gtcggcgtcg cagcgggcct 6360
ggcgggggcg gtttccatgg cgttcggaac cgtgctgacc cgcaagtggc aacctcccgt 6420
gcctctgctc acctttaccg cctggcaact ggcggccgga ggacttctgc tcgttccagt 6480
agctttagtg tttgatccgc caatcccgat gcctacagga accaatgttc tcggcctggc 6540
gtggctcggc ctgatcggag cgggtttaac ctacttcctt tggttccggg ggatctcgcg 6600
actcgaacct acagttgttt ccttactggg ctttctcagc cgggatggcg ctaagaagct 6660

attgccgccg atcttcatat gcggtgtgaa ataccgcaca gatgcgtaag gagaaaatac 6720
cgcatcaggc gctcttccgc ttcctcgctc actgactcgc tgcgctcggt cgttcggctg 6780
cggcgagcgg tatcagctca ctcaaaggcg gtaatacggt tatccacaga atcaggggat 6840
aacgcaggaa agaacatgtg agcaaaaggc cagcaaaagg ccaggaaccg taaaaaggcc 6900
gcgttgctgg cgtttttcca taggctccgc ccccctgacg agcatcacaa aaatcgacgc 6960
tcaagtcaga ggtggcgaaa cccgacagga ctataaagat accaggcgtt tccccctgga 7020
agctccctcg tgcgctctcc tgttccgacc ctgccgctta ccggatacct gtccgccttt 7080
ctcccttcgg gaagcgtggc gctttctcaa tgctcacgct gtaggtatct cagttcggtg 7140
taggtcgttc gctccaagct gggctgtgtg cacgaacccc ccgttcagcc cgaccgctgc 7200
gccttatccg gtaactatcg tcttgagtcc aacccggtaa gacacgactt atcgccactg 7260
gcagcagcca ctggtaacag gattagcaga gcgaggtatg taggcggtgc tacagagttc 7320
ttgaagtggt ggcctaacta cggctacact agaaggacag tatttggtat ctgcgctctg 7380
ctgaagccag ttaccttcgg aaaaagagtt ggtagctctt gatccggcaa acaaaccacc 7440
gctggtagcg gtggtttttt tgtttgcaag cagcagatta cgcgcagaaa aaaaggatat 7500
caagaagatc ctttgatctt ttctacgggg tctgacgctc agtggaacga aaactcacgt 7560
taagggattt tggtcatgag attatcaaaa aggatcttca cctagatcct tttaaattaa 7620
aaatgaagtt ttaaatcaat ctaaagtata tatgagtaaa cttggtctga cagttaccaa 7680
tgcttaatca gtgaggcacc tatctcagcg atctgtctat ttcgttcatc catagttgcc 7740
tgactccccg tcgtgtagat aactacgata cgggagggct taccatctgg ccccagtgct 7800
gcaatgatac cgcgagaccc acgctcaccg gctccagatt tatcagcaat aaaccagcca 7860
gccggaaggg ccgagcgcag aagtggtcct gcaactttat ccgcctccat ccagtctatt 7920
aaacaagtgg cagcaacgga ttcgcaaacc tgtcacgcct tttgtgccaa aagccgcgcc 7980
aggtttgcga tccgctgtgc caggcgttag gcgtcatatg aagatttcgg tgatccctga 8040
gcaggtggcg gaaacattgg atgctgagaa ccatttcatt gttcgtgaag tgttcgatgt 8100
gcacctatcc gaccaaggct ttgaactatc taccagaagt gtgagcccct accggaagga 8160
ttacatctcg gatgatgact ctgatgaaga ctctgcttgc tatggcgcat tcatcgacca 8220
agagcttgtc gggaagattg aactcaactc aacatggaac gatctagcct ctatcgaaca 8280
cattgttgtg tcgcacacgc accgaggcaa aggagtcgcg cacagtctca tcgaatttgc 8340
gaaaaagtgg gcactaagca gacagctcct tggcatacga ttagagacac aaacgaacaa 8400
tgtacctgcc tgcaatttgt acgcaaaatg tggctttact ctcggcggca ttgacctgtt 8460
cacgtataaa actagacctc aagtctcgaa cgaaacagcg atgtactggt actggttctc 8520

gggagcacag gatgacgcct aacaattcat tcaagccgac accgcttcgc ggcgcggctt 8580
aattcaggag ttaaacatca tgagggaagc ggtgatcgcc gaagtatcga ctcaactatc 8640
agaggtagtt ggcgtcatcg agcgccatct cgaaccgacg ttgctggccg tacatttgta 8700
cggctccgca gtggatggcg gcctgaagcc acacagtgat attgatttgc tggttacggt 8760
gaccgtaagg cttgatgaaa caacgcggcg agctttgatc aacgaccttt tggaaacttc 8820
ggcttcccct ggagagagcg agattctccg cgctgtagaa gtcaccattg ttgtgcacga 8880
cgacatcatt ccgtggcgtt atccagctaa gcgcgaactg caatttggag aatggcagcg 8940
caatgacatt cttgcaggta tcttcgagcc agccacgatc gacattgatc tggctatctt 9000
gctgacaaaa gcaagagaac atagcgttgc cttggtaggt ccagcggcgg aggaactctt 9060
tgatccggtt cctgaacagg atctatttga ggcgctaaat gaaaccttaa cgctatggaa 9120
ctcgccgccc gactgggctg gcgatgagcg aaatgtagtg cttacgttgt cccgcatttg 9180
gtacagcgca gtaaccggca aaatcgcgcc gaaggatgtc gctgccgact gggcaatgga 9240
gcgcctgccg gcccagtatc agcccgtcat acttgaagct aggcaggctt atcttggaca 9300
agaagatcgc ttggcctcgc gcgcagatca gttggaagaa tttgttcact acgtgaaagg 9360
cgagatcacc aaggtagtcg gcaaataatg tctaacaatt cgttcaagcc gacgccgctt 9420
cgcggcgcgg cttaactcaa gcgttagaga gctggggaag actatgcgcg atctgttgaa 9480
ggtggttcta agcctcgtac ttgcgatggc atcggggcag gcacttgctg acctgccaat 9540
tgttttagtg gatgaagctc gtcttcccta tgactactcc ccatccaact acgacatttc 9600
tccaagcaac tacgacaact ccataagcaa ttacgacaat agtccatcaa attacgacaa 9660
ctctgagagc aactacgata atagttcatc caattacgac aatagtcgca acggaaatcg 9720
taggcttata tatagcgcaa atgggtctcg cactttcgcc ggctactacg tcattgccaa 9780
caatgggaca acgaacttct tttccacatc tggcaaaagg atgttctaca ccccaaaagg 9840
ggggcgcggc gtctatggcg gcaaagatgg gagcttctgc ggggcattgg tcgtcataaa 9900
tggccaattt tcgcttgccc tgacagataa cggcctgaag atcatgtatc taagcaacta 9960
gcctgctctc taataaaatg ttaggagctt ggctgccatt tttggggtga ggccgttcgc 10020
ggccgagggg cgcagcccct gggggggatgg gaggcccgcg ttagcgggcc gggagggttc 10080
gagaaggggg ggcacccccc ttcggcgtgc gcggtcacgc gccagggcgc agccctggtt 10140
aaaaacaagg tttataaata ttggtttaaa agcaggttaa aagacaggtt agcggtggcc 10200
gaaaaacggg cggaaaccct tgcaaatgct ggattttctg cctgtggaca gcccctcaaa 10260
tgtcaatagg tgcgcccctc atctgtcagc actctgcccc tcaagtgtca aggatcgcgc 10320
ccctcatctg tcagtagtcg cgcccctcaa gtgtcaatac cgcagggcac ttatccccag 10380

gcttgtccac atcatctgtg ggaaactcgc gtaaaatcag gcgttttcgc cgatttgcga 10440
ggctggccag ctccacgtcg ccggccgaaa tcgagcctgc ccctcatctg tcaacgccgc 10500
gccgggtgag tcggcccctc aagtgtcaac gtccgcccct catctgtcag tgagggccaa 10560
gttttccgcg aggtatccac aacgccggcg gccggccgcg gtgtctcgca cacggcttcg 10620
acggcgtttc tggcgcgttt gcagggccat agacggccgc cagcccagcg gcgagggcaa 10680
ccagcccggt gagcgtcgga aagggtcgac atcttgctgc gttcggatat tttcgtggag 10740
ttcccgccac agacccggat tgaaggcgag atccagcaac tcgcgccaga tcatcctgtg 10800
acggaacttt ggcgcgtgat gactggccag gacgtcggcc gaaagagcga caagcagatc 10860
acgattttcg acagcgtcgg atttgcgatc gaggattttt cggcgctgcg ctacgtccgc 10920
gaccgcgttg agggatcaag ccacagcagc ccactcgacc ttctagccga cccagacgag 10980
ccaagggatc tttttggaat gctgctccgt cgtcaggctt tccgacgttt gggtggttga 11040
acagaagtca ttatcgtacg gaatgccagc actcccgagg ggaaccctgt ggttggcatg 11100
cacatacaaa tggacgaacg gataaacctt ttcacgccct tttaaatatc cgttattcta 11160
ataaacgctc ttttctctta ggtttacccg ccaatatatc ctgtcaaaca ctgatagttt 11220
aaactgaagg cgggaaacga caatctgatc atgagcggag aattaaggga gtcacgttat 11280
gacccccgcc gatgacgcgg gacaagccgt tttacgtttg gaactgacag aaccgcaacg 11340
attgaaggag ccactcagcc ccaatacgca aaccgcctct ccccgcgcgt tggccgattc 11400
attaatgcag ctggcacgac aggtttcccg actggaaagc gggcagtgag cgcaacgcaa 11460
ttaatgtgag ttagctcact cattaggcac cccaggcttt acactttatg cttccggctc 11520
gtatgttgtg tggaattgtg agcggataac aatttcacac aggaaacagc tatgaccatg 11580
attacgccaa gctatttagg tgacactata gaatactcaa gctatgcatc caacgcgttg 11640
ggagctctcc catatcgacc tgcaggcggc cgctcgacga attaattcca atcccacaaa 11700
aatctgagct taacagcaca gttgctcctc tcagagcaga atcgggtatt caacaccctc 11760
atatcaacta ctacgttgtg tataacggtc cacatgccgg tatatacgat gactggggtt 11820
gtacaaaggc ggcaacaaac ggcgttcccg gagttgcaca caagaaattt gccactatta 11880
cagaggcaag agcagcagct gacgcgtaca caacaagtca gcaaacagac aggttgaact 11940
tcatccccaa aggagaagct caactcaagc ccaagagctt tgctaaggcc ctaacaagcc 12000
caccaaagca aaaagcccac tggctcacgc taggaaccaa aaggcccagc agtgatccag 12060
ccccaaaaga gatctccttt gccccggaga ttacaatgga cgatttcctc tatctttacg 12120
atctaggaag gaagttcgaa ggtgaaggtg acgacactat gttcaccact gataatgaga 12180
aggttagcct cttcaatttc agaaagaatg ctgacccaca gatggttaga gaggcctacg 12240

cagcaggtct catcaagacg atctacccga gtaacaatct ccaggagatc aaataccttc 12300

ccaagaaggt taaagatgca gtcaaaagat tcaggactaa ttgcatcaag aacacagaga 12360

aagacatatt tctcaagatc agaagtacta ttccagtatg gacgattcaa ggcttgcttc 12420

ataaaccaag gcaagtaata gagattggag tctctaaaaa ggtagttcct actgaatcta 12480

aggccatgca tggagtctaa gattcaaatc gaggatctaa cagaactcgc cgtgaagact 12540

ggcgaacagt tcatacagag tcttttacga ctcaatgaca agaagaaaat cttcgtcaac 12600

atggtggagc acgacactct ggtctactcc aaaaatgtca aagatacagt ctcagaagac 12660

caaagggcta ttgagacttt tcaacaaagg ataatttcgg gaaacctcct cggattccat 12720

tgcccagcta tctgtcactt catcgaaagg acagtagaaa aggaaggtgg ctcctacaaa 12780

tgccatcatt gcgataaagg aaaggctatc attcaagatc tctctgccga cagtggtccc 12840

aaagatggac ccccacccac gaggagcatc gtggaaaaag aagacgttcc aaccacgtct 12900

tcaaagcaag tggattgatg tgacatctcc actgacgtaa gggatgacgc acaatcccac 12960

tatccttcgc aagacccttc ctctatataa ggaagttcat ttcatttgga gaggacacgc 13020

tcgagacaag tttgtacaaa aaagctgaac gagaaacgta aaatgatata aatatcaata 13080

tattaaatta gattttgcat aaaaaacaga ctacataata ctgtaaaaca caacatatcc 13140

agtcactatg aatcaactac ttagatggta ttagtgacct gtagtcgacc gacagccttc 13200

caaatgttct tcgggtgatg ctgccaactt agtcgaccga cagccttcca aatgttcttc 13260

tcaaacggaa tcgtcgtatc cagcctactc gctattgtcc tcaatgccgt attaaatcat 13320

aaaaagaaat aagaaaaaga ggtgcgagcc tcttttttgt gtgacaaaat aaaaacatct 13380

acctattcat atacgctagt gtcatagtcc tgaaaatcat ctgcatcaag aacaatttca 13440

caactcttat acttttctct tacaagtcgt tcggcttcat ctggattttc agcctctata 13500

cttactaaac gtgataaagt ttctgtaatt tctactgtat cgacctgcag actggctgtg 13560

tataagggag cctgacattt atattcccca gaacatcagg ttaatggcgt ttttgatgtc 13620

attttcgcgg tggctgagat cagccacttc ttccccgata acggagaccg gcacactggc 13680

catatcggtg gtcatcatgc gccagctttc atccccgata tgcaccaccg ggtaaagttc 13740

acgggagact ttatctgaca gcagacgtgc actggccagg gggatcacca tccgtcgccc 13800

gggcgtgtca ataatatcac tctgtacatc cacaaacaga cgataacggc tctctctttt 13860

ataggtgtaa accttaaact gcatttcacc agtccctgtt ctcgtcagca aaagagccgt 13920

tcatttcaat aaaccgggcg acctcagcca tcccttcctg attttccgct ttccagcgtt 13980

cggcacgcag acgacgggct tcattctgca tggttgtgct taccagaccg gagatattga 14040

catcatatat gccttgagca actgatagct gtcgctgtca actgtcactg taatacgctg 14100

cttcatagca cacctctttt tgacatactt cgggtagtgc cgatcaacgt ctcattttcg 14160
ccaaaagttg gcccagggct tcccggtatc aacagggaca ccaggattta tttattctgc 14220
gaagtgatct tccgtcacag gtatttattc ggcgcaaagt gcgtcgggtg atgctgccaa 14280
cttagtcgac tacaggtcac taataccatc taagtagttg attcatagtg actggatatg 14340
ttgtgtttta cagtattatg tagtctgttt tttatgcaaa atctaattta atatattgat 14400
atttatatca ttttacgttt ctcgttcagc tttcttgtac aaagtggtct cgaggaattc 14460
ggtaccccag cttggtaagg aaataattat tttctttttt ccttttagta taaaatagtt 14520
aagtgatgtt aattagtatg attataataa tatagttgtt ataattgtga aaaaataatt 14580
tataaatata ttgtttacat aaacaacata gtaatgtaaa aaaatatgac aagtgatgtg 14640
taagacgaag aagataaaag ttgagagtaa gtatattatt tttaatgaat ttgatcgaac 14700
atgtaagatg atatactagc attaatattt gttttaatca taatagtaat tctagctggt 14760
ttgatgaatt aaatatcaat gataaaatac tatagtaaaa ataagaataa ataaattaaa 14820
ataatatttt tttatgatta atagtttatt atataattaa atatctatac cattactaaa 14880
tattttagtt taaaagttaa taaatatttt gttagaaatt ccaatctgct tgtaatttat 14940
caataaacaa aatattaaat aacaagctaa agtaacaaat aatatcaaac taatagaaac 15000
agtaatctaa tgtaacaaaa cataatctaa tgctaatata acaaagcgca agatctatca 15060
ttttatatag tattattttc aatcaacatt cttattaatt tctaaataat acttgtagtt 15120
ttattaactt ctaaatggat tgactattaa ttaaatgaat tagtcgaaca tgaataaaca 15180
aggtaacatg atagatcatg tcattgtgtt atcattgatc ttacatttgg attgattaca 15240
gttgggaagc tgggttcgaa atcgataagc ttgcgctgca gttatcatca tcatcataga 15300
cacacgaaat aaagtaatca gattatcagt taaagctatg taatatttgc gccataacca 15360
atcaattaaa aaatagatca gtttaaagaa agatcaaagc tcaaaaaaat aaaaagagaa 15420
aagggtccta accaagaaaa tgaaggagaa aaactagaaa tttacctgca caagcttgga 15480
tcctctagac cactttgtac aagaaagctg aacgagaaac gtaaaatgat ataaatatca 15540
atatattaaa ttagattttg cataaaaaac agactacata atactgtaaa acacaacata 15600
tccagtcact atgaatcaac tacttagatg gtattagtga cctgtagtcg actaagttgg 15660
cagcatcacc cgacgcactt tgcgccgaat aaatacctgt gacggaagat cacttcgcag 15720
aataaataaa tcctggtgtc cctgttgata ccgggaagcc ctgggccaac ttttggcgaa 15780
aatgagacgt tgatcggatt tcacaactct tatacttttc tcttacaagt cgttcggctt 15840
catctggatt ttcagcctct atacttacta aacgtgataa agtttctgta atttctactg 15900
tatcgacctg cagactggct gtgtataagg gagcctgaca tttatattcc ccagaacatc 15960

aggttaatgg cgtttttgat gtcattttcg cggtggctga gatcagccac ttcttccccg 16020
ataacggaga ccggcacact ggccatatcg gtggtcatca tgcgccagct ttcatccccg 16080
atatgcacca ccgggtaaag ttcacgggag actttatctg acagcagacg tgcactggcc 16140
aggggggatca ccatccgtcg cccgggcgtg tcaataatat cactctgtac atccacaaac 16200
agacgataac ggctctctct tttataggtg taaaccttaa actgcatttc accagtccct 16260
gttctcgtca gcaaaagagc cgttcatttc aataaaccgg gcgacctcag ccatcccttc 16320
ctgattttcc gctttccagc gttcggcacg cagacgacgg gcttcattct gcatggttgt 16380
gcttaccaga ccggagatat tgacatcata tatgccttga gcaactgata gctgtcgctg 16440
tcaactgtca ctgtaatacg ctgcttcata gcacacctct ttttgacata cttctgttct 16500
tgatgcagat gattttcagg actatgacac tagcgtatat gaataggtag atgtttttat 16560
tttgtcacac aaaaaagagg ctcgcacctc tttttcttat ttctttttat gatttaatac 16620
ggcattgagg acaatagcga gtaggctgga tacgacgatt ccgtttgaga agaacatttg 16680
gaaggctgtc ggtcgactaa gttggcagca tcacccgaag aacatttgga aggctgtcgg 16740
tcgactacag gtcactaata ccatctaagt agttgattca tagtgactgg atatgttgtg 16800
ttttacagta ttatgtagtc tgttttttat gcaaaatcta atttaatata ttgatattta 16860
tatcatttta cgtttctcgt tcagcttttt tgtacaaact tgtctagagt cctgctttaa 16920
tgagatatgc gagacgccta tgatcgcatg atatttgctt tcaattctgt tgtgcacgtt 16980
gtaaaaaacc tgagcatgtg tagctcagat ccttaccgcc ggtttcggtt cattctaatg 17040
aatatatcac ccgttactat cgtattttta tgaataatat tctccgttca atttactgat 17100
tgtaccctac tacttatatg tacaatatta aaatgaaaac aatatattgt gctgaatagg 17160
tttatagcga catctatgat agagcgccac aataacaaac aattgcgttt tattattaca 17220
aatccaattt taaaaaaagc ggcagaaccg gtcaaaccta aaagactgat tacataaatc 17280
ttattcaaat ttcaaaaggc cccaggggct agtatctacg acacaccgag cggcgaacta 17340
ataacgttca ctgaagggaa ctccggttcc ccgccggcgc gcatgggtga gattccttga 17400
agttgagtat tggccgtccg ctctaccgaa agttacgggc accattcaac ccggtccagc 17460
acggcggccg ggtaaccgac ttgctgcccc gagaattatg cagcattttt ttggtgtatg 17520
tgggccccaa atgaagtgca ggtcaaacct tgacagtgac gacaaatcgt tgggcgggtc 17580
cagggcgaat tttgcgacaa catgtcgagg ctcagcagga cctgcaggca tgcaagctag 17640
cttactagtg atgcatattc tatagtgtca cctaaatctg c 17681

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/00073

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 15/09 15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

SEE ELECTRONIC DATABASE BOX BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SEE ELECTRONIC DATABASE BOX BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, Chem Abs, Biosis, WPIDS
keywords: gene silencing, genetic vector, dsRNA, multiple recombination sites, recombinatorial cloning, RNAi
GenBank, EMBL: sequence IDs 13, 23-26

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	WESLEY, S. V. et al (2001) "Construct design for efficient, effective and high-throughput gene silencing in plants" *The Plant Journal*, 27(6), 581-590.	1-33
A	MONTGOMERY, M. K. et al (1998) "Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression" *TIG* 14(7), 255-258	
A	AU-A-43685/99 (Novartis AG) 2 December 1999	

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 2002	04 MAR 2002

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	PHILIPPA WYRDEMAN Telephone No : (02) 6283 2554

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU02/00073

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	43685/99	WO	99/61631	BR	9910729	EP	1080208
HU	0102103	PL	344312				

END OF ANNEX